The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria

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The aim of this investigation was to perform an extensive screening using HPLC and ^{13}C -NMR spectroscopy to disclose the spectrum of osmolytes produced by aerobic heterotrophic and anoxygenic phototrophic eubacteria. The most predominant solutes detected within a wide range of marine and halophilic micro-organisms were two recently discovered tetrahydropyrimidines ectoine and hydroxyectoine, which were synthesized in response to osmotic stress.

Introduction

Halophilic and halotolerant micro-organisms can be isolated from various saline biotops such as marine sediments, solar salterns, salt lakes and salt mines (Trüper & Galinski, 1986). Even organisms from habitats of high water activity (fresh water) occasionally exhibit surprisingly high levels of osmotic tolerance (Kanemasa *et al.*, 1972; Komarata & Kates, 1975). A variety of possible applications of these organisms including food processing microbiology, waste water treatment, production of commercially useful compounds like PHB, enzymes, antibiotics, polyols and amino acids and, most recently, as a source for enzyme protectants have been demonstrated (for reviews, see Austin, 1989; Okami, 1986; Ben-Amotz & Avron, 1983; Staley & Stanley, 1986; Galinski & Tindall, 1991).

Halophilic and halotolerant micro-organisms are distributed among several phylogenetic taxa and two different strategies to reduce intracellular water activity have been evolved. Archaeobacteria and anaerobic heterotrophic eubacteria accumulate inorganic ions such as K^+ , Na^+ , Cl^- (Oren, 1985; Rengpipat *et al.*, 1988), except for the methanogens where recent investigations have demonstrated the occurrence of new organic solutes (Robertson *et al.*, 1990a, b; Lai *et al.*, 1991). The other groups of halophiles including phototrophic and aerobic chemoheterotrophic eubacteria, yeasts, algae and fungi mainly accumulate organic compounds termed com-

patible solutes (Brown, 1976). Polyols were reported from eukaryotic algae and fungi (Avron, 1986; Wegmann, 1986; Jovall *et al.*, 1990; Van Eck *et al.*, 1989). Glycine betaine and sugars including sugar derivatives were predominantly found in phototrophic eubacteria (Galinski & Trüper, 1982; Mackay *et al.*, 1984). The accumulation of glycine betaine originally reported from aerobic heterotrophic eubacteria (Imhoff & Rodriguez-Valera, 1984) was subsequently shown to result from growth media supplements (yeast extract) containing glycine betaine and/or suitable precursors (Wohlfarth *et al.*, 1990).

Investigations into the *de novo* synthesis of compatible solutes have so far concentrated on non-halophilic bacteria like *Escherichia coli* and *Rhizobium meliloti* (Larsen *et al.*, 1987; Smith & Smith, 1989), where the accumulation of glutamate and trehalose were observed, whilst proline and betaine were only used as osmotica when supplemented in the media (Grothe *et al.*, 1986; Le Rudulier & Bernard, 1986; Smith *et al.*, 1988). Biosynthesis of proline under osmotic stress has only been reported for some halotolerant *Bacillus* species (Tempest *et al.*, 1970; Whatmore *et al.*, 1990). All other investigations into the osmoregulation of halophilic aerobic heterotrophic eubacteria except for the *Halomonadaceae* (Wohlfarth *et al.*, 1990) have so far failed to detect sufficiently high concentrations of osmolytes (organic and inorganic). This apparent lack of solutes stimulated us to look for so far undetected natural substances using ^{13}C -NMR spectroscopy and HPLC. To assess the variety of compatible solutes in nature we have conducted a complete survey on nearly all halophilic strains of

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Table 1. Compatible solutes detectable in cell extracts of halophiles by ^{13}C -NMR spectroscopy

The dominant solute present at the experimental salinity is emphasized (+ +). Other detectable solutes are marked with (+), while those solutes that never occur as primary osmolytes are listed in the column 'others'. Organisms are summarized according to the classification of Woese (1987). The predominance of ectoines in chemoheterotrophic eubacteria (grown on GM media) and of glycine betaine in anoxygenic phototrophic bacteria is obvious. Abbreviations: ATCC, American Type Culture Collection, Rockville, MD, USA; CCM, Czechoslovak Collection of Micro-organisms, Brno, Czechoslovakia; DSM, Deutsche Sammlung von Mikroorganismen, Darmstadt, FRG; GM, glucose-mineral medium; YE, yeast extract containing complex medium; PM, medium for phototrophic bacteria; ala, alanine; gg, glucosylglycerol; gluc, glucose; gl, glutamate; β -gl, β -glutamate; ga, *N*-acetylglutaminylglutamine amide; cga, α -*N*-carbamoyl-L-glutamine amide; δ , not yet identified.

Organism	Medium	Temp. (° C)	Salinity (‰ w/v)	Solutes					
				Betaine	Ectoine	Hydroxy- ectoine	Tre- halose	Sucrose	Others
<i>Firmicutes</i>									
<i>Actinopolyspora halophila</i> (ATCC 27976 ^T)	GM	37	25	++			+		gluc
<i>Actinomyce</i> te A5-1 (<i>Nocardiopsis</i> sp.)	GM	35	10		++	+	+		gl, β -gl
<i>Marinococcus halophilus</i> (DSM 20408 ^T)	GM	30	10		++	+			gluc
<i>Micrococcus halobius</i> (DSM 20541 ^T)	GM	30	10		++	+	+		gluc, gl
<i>Micrococcus varians</i> var. <i>halophilus</i> (CCM 3316)*	GM	30	10		++	+	+		gluc, gl
<i>Streptomyces griseolus</i> (DSM 40067 ^T)	YE	30	5	+		++	+		
M 52 (coccal isolate)*	GM	37	15			++			gluc, ala
<i>Proteobacteria</i> (α -subclass)									
<i>Rhodobacter sulfidophilus</i> (W 4 = DSM 1374 ^T)†	PM	30	10		++				gg
<i>Rhodopseudomonas marina</i> (BN 125 = DSM 2780 ^T)†	PM	30	7.5				++		ga
<i>Rhodospirillum salexigens</i> (DSM 2132 ^T)†	PM	30	15	++					
<i>Rhodospirillum salinarum</i> (BN 40)†	PM	30	20	++	+				
<i>Proteobacteria</i> (α -subclass)									
<i>Chromatium purpuratum</i> (DSM 1591 ^T)†	PM	30	7.5	+				++	ga
<i>Chromatium salexigens</i> (SG 3201 = DSM 4395 ^T)†	PM	30	7.5	++				+	ga
<i>Ectothiorhodospira marismortui</i> (EG 1 = DSM 4180 ^T)†	PM	35	15	++				+	cga
<i>Ectothiorhodospira halochloris</i> (BN 9850 = DSM 1059 ^T)†	PM	40	20	++	+		+		
<i>Ectothiorhodospira halophila</i> (DSM 244 ^T)†	PM	40	20	++	+		+		
<i>Ectothiorhodospira abdelmalekii</i> (BN 9840 = DSM 2110 ^T)†	PM	40	20	++	+		+		
<i>Thicapsa halophila</i> (SG 3202 = DSM 6210 ^T)†‡	PM	30	10	++				+	δ , gl, β -gl
<i>Deleya halophila</i> (CCM 3662 ^T)	GM	30	10		++	+			gl
<i>Halomonas elongata</i> (ATCC 33173 ^T)	GM	40	20		++	+			gl, gluc
<i>Halomonas halmophila</i> (CCM 2833 ^T)	GM	30	10		++	+			gl, ala
<i>Paracoccus halodenitrificans</i> (DSM 735 ^T)§	GM	30	10		++				gluc, gl
<i>Vibrio alginolyticus</i> (DSM 2171 ^T)	GM	30	10		++				gl
<i>Vibrio costicola</i> (CCM 2811)	GM	30	10		++				gl
<i>Chromohalobacter marismortui</i> (ATCC 17056)	YE	30	10	+	++				

* Taxonomic position uncertain (B. J. Tindall, personal communication).

† Phototrophic bacteria.

‡ Caumette *et al.* (1991).

§ Atypical *Paracoccus*, possibly γ -subclass (B. J. Tindall, personal communication).

|| Systematic position uncertain, probably γ -subclass (Ventosa *et al.*, 1989).

aerobic heterotrophic eubacteria available from culture collections (Table 1) and in addition we have also screened approximately 450 of our own isolates from a wide range of saline biotops (Wohlfarth *et al.*, 1989). Strains were usually grown both on complex and synthetic media to distinguish between *de novo* synthesis

of solutes and accumulation from the medium and/or conversion from suitable precursors. For reasons of comparison, results obtained for phototrophic organisms (Galinski, 1986) are also included owing to the close relationship of anoxygenic phototrophic and heterotrophic eubacteria (Woese, 1987; Trüper *et al.*, 1991).

Methods

The organisms from strain collections used in our study are listed in Tables 1 and 2. Heterotrophs, with the exception of *Halomonadaceae* (Wohlfarth *et al.*, 1990), were grown in medium K10 or G10. Complex medium K10 consisted of (g l⁻¹): NaCl (90), artificial sea salt (10), yeast extract (Oxoid) (7.5) and caseine hydrolysate (Oxoid) (7.5). Glucose-mineral medium G10 consisted of (g l⁻¹): NaCl (90), artificial sea salt (10; DSM, Catalogue of strains 1989, Medium 79), NH₄Cl (2.0), KH₂PO₄ (0.5), FeSO₄·7H₂O (0.01), glucose (10.0), yeast extract (0.1) and vitamin solution VA (1 ml l⁻¹; Imhoff & Trüper, 1977). Glucose and phosphate solutions were autoclaved separately, the vitamin solution was sterilized by filtration. Betaine medium B10 differed from G10 by the exchange of glucose with glycine betaine (10 g). For shaking cultures these media were buffered with 50 mM-Tris/HCl (pH 7.5).

Media for phototrophic *Ectothiorhodospiraceae* are described by Galinski & Trüper (1982) and Oren *et al.* (1989), and for *Chromatiaceae* in Caumette *et al.* (1988). Cell material of *Rhodospirillaceae* was kindly supplied by Dr J. F. Imhoff, Institut Für Mikrobiologie & Biotechnologie, Bonn. Cultivation and cell harvest of phototrophic bacteria was carried out according to Galinski & Herzog (1990).

Samples for the isolation of halophilic aerobic eubacteria were taken from various saline environments. These sampling sites included: Agadir, Morocco (brackish and coastal waters); Ile d'Oleron, France (coastal water and sediment); Island of Kos, Greece (solar saltern); Schott el Dscherid, Tunisia (athalassohaline salt lake); Alicante, Spain (solar saltern, water, sediments and hypersaline soils); Bad Aussee, Austria (salt mine waters); and Bad Salzuffen, Germany ('Gradierwerk', waters and crusts).

To assess the natural distribution of halophilic eubacteria capable of *de novo* synthesis of compatible solutes, growth media with defined

Table 2. HPLC quantification of osmolytes from strains synthesizing ectoines

The strains were grown in rotary flasks at a salinity of 10% salts and 30 °C. The solute content is quantified in terms of µmol or µg (mg dry wt)⁻¹ and was found to range between 0.6 and 1.3 µmol (mg dry wt)⁻¹, thus accounting for 10–20% of the cellular dry weight. Growth rates on G10 medium were determined to select effective producer strains for both ectoines. The use of glycine betaine as osmolyte, carbon and energy source is common in some halophilic eubacteria as indicated in the corresponding column. +/–, Growth/no growth detected.

Organism	Medium	µmol (mg dry wt) ⁻¹						Un-identified peak area (%)	Total solutes			Growth on betaine as sole C-source
		Betaine	Ectoine	Hydroxy-ectoine	Alanine	Trehalose	Glucose		µmol (mg dry wt) ⁻¹	µg (mg dry wt) ⁻¹	µ _{max} (h ⁻¹)	
<i>Chromohalobacter marismortui</i> (ATCC 17056)	G10*											ND
	K10	0.462	0.556	–	–	–	–	0	1.018	133.1	ND	
<i>Deleya halophila</i> (CCM 3662)	G10	–	0.735	0.066	0.143	–	0.089	0	1.033	143.6	0.206	+
	K10	0.431	0.569	–	–	0.024	0.015	0	1.039	142.2	ND	
<i>Halomonas elongata</i> (ATCC 33173)	G10	–	0.762	0.036	–	–	0.162	0	1.96	143.1	0.242	+
	K10	0.231	0.745	–	–	–	–	0	0.976	132.9	ND	
<i>Halomonas halmophila</i> (CCM 2833)	G10	–	0.710	0.042	–	–	0.052	0	0.804	116.9	0.129	+
	K10	0.434	0.389	–	0.037	0.138	0.045	0	1.043	164.7	ND	
<i>Halovibrio variabilis</i> (DSM 3051 ^T)	G10†											ND
	K10	0.282	0.268	0.334	–	0.013	–	0	0.897	128.7	ND	
M 52 (coccal isolate)	G10	–	0.161	0.576	–	–	0.166	0	0.903	144.4	0.062	–
	K10	0.731	0.135	0.162	–	0.040	–	1.3	> 1.068	> 144.3	ND	
<i>Marinococcus albus</i> (DSM 20748 ^T)	G10	–	0.578	0.049	0.286	–	0.182	0	1.095	148.1	0.068	–
	K10	0.631	0.127	–	0.104	0.004	–	20.4	> 0.866	> 102.6	ND	
<i>Marinococcus halophilus</i> (DSM 20408 ^T)	G10	–	0.485	0.354	–	–	0.153	0	0.992	152.7	0.065	–
	K10	1.111	0.027	0.142	–	–	–	0	1.279	156.6	ND	
<i>Micrococcus halobius</i> (DSM 20541 ^T)	G10	–	0.473	0.124	–	0.242	0.182	0	1.021	202.5	0.066	+
	K10	0.310	0.661	0.043	–	0.080	0.067	0	1.161	176.5	ND	
<i>Micrococcus varians</i> var. <i>halophilus</i> (CCM 3316)	G10	–	0.350	0.209	–	0.224	0.106	0	0.889	178.7	0.093	–
	K10	0.270	0.662	0.008	–	0.011	–	0	0.951	130.7	ND	
<i>Paracoccus halodenitrificans</i> (DSM 735 ^T)	G10	–	0.624	–	–	–	0.119	0	0.743	110.1	0.081	–
	K10	0.288	0.550	–	–	0.146	–	0	0.984	161.8	ND	
<i>Pseudomonas halophila</i> (DSM 3050 ^T)	G10†											ND
	K10	0.616	0.052	0.176	–	–	–	8.5	0.844	107.5	ND	
<i>Pseudomonas halosaccharolytica</i> (CCM 2851)	G10	–	0.568	0.027	0.147	–	0.108	0	0.850	117.5	0.151	+
	K10	0.393	0.489	–	–	–	–	0	0.882	115.5	ND	
<i>Vibrio alginolyticus</i> (DSM 2171 ^T)	G10	–	0.532	–	0.093	–	0.104	0	0.729	102.6	0.124	–
	K10	0.347	0.453	–	0.057	–	0.053	4.5	> 0.91	> 119.6	ND	
<i>Vibrio costicola</i> (CCM 2811)	G10	–	0.539	–	–	–	0.067	0	0.606	88.6	0.074	–
	K10	0.205	0.612	–	–	–	–	0	0.817	110.9	ND	

ND, Not determined.

* Insufficient growth in G10 liquid medium.

† Glucose is not a suitable substrate; however, when grown on MEI-glycerol media (Fendrich, 1988) ectoines are the predominant solutes.

carbon sources were used for the screening procedure. Our isolation media contained varying and constitutive ingredients. The basal medium consisted of (g l^{-1}): NH_4Cl (2.0), K_2HPO_4 (0.5), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01), NaBr (0.1), KCl (2.0), Tris (6.0) titrated with HCl to pH 7.2. The total salinity ranged from 10 up to 25%. The concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ varied between 0.1 and 20 g, that of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ between 0 and 5.0 g. For synthetic mineral media the following compounds were chosen as carbon sources: glucose, trehalose, glycerol, malate, glutamate, glycine betaine, acetate, aspartate, cellulose and starch. Some of the media were supplemented with yeast extract (0.1 g l^{-1}). Samples were cooled, stored and subsequently plated on saline agar plates (10–20%, w/v, NaCl) and incubated at various temperatures (25–40 °C). Organisms forming colonies in less than one week were isolated, morphologically characterized and further investigated.

Further methods (culture conditions, cell harvest, HPLC analysis of compatible solutes and NMR spectroscopy) were the same as described previously (Wohlfarth *et al.*, 1990). As a simplification of the sample preparation procedure for HPLC described previously (Galinski & Herzog, 1990), we extracted 30 mg freeze-dried cell material by rapid shaking with 1 ml $\text{CHCl}_3/\text{H}_2\text{O}$ (1:1, v/v).

NMR measurements were performed on a Bruker WP 90 and a Varian XL 300 unit using D_2O as a lock signal and trimethylsilylpropionic acid sodium salt (TMSP) as internal standard.

Results

Screening for heterotrophic halophilic eubacteria and their osmolytes

We isolated approximately 450 cultures from the specified saline habitats. Only those isolates forming colonies within one week were chosen for further studies. The isolates were superficially characterized by morphological features such as colony form and colour, cell shape and motility. Gram-negative rods constituted 55% of the isolates, 15% were spore-forming rods, 10% cocci, 10% vibrio-shaped organisms, 5% halobacteria, 4% actinomycetes, 1% spirilla and 2% of irregular shape. Most actinomycetes were isolated on starch and cellulose media, while spirilla were only found on media containing glutamate as the carbon source.

Of those strains displaying similar characteristics, representatives were selected randomly for cultivation in liquid media. Approximately 10% of the strains that grew well on agar plates could not be cultivated in liquid media. Finally, cell material derived from 150 strains was subjected to HPLC determination of compatible solutes. With only a few exceptions, HPLC analysis revealed the presence of organic molecules. In about 90% of the investigated strains, refractive index monitoring revealed a predominant compound with a retention time identical to ectoine (Fig. 1) originally characterized in a phototrophic eubacterium (Galinski *et al.*, 1985) and subsequently described in some heterotrophic eubacteria (Wohlfarth *et al.*, 1989; Regev *et al.*, 1990).

Fig. 1. The tetrahydropyrimidines: ectoine ($\text{R} = \text{H}$); hydroxyectoine ($\text{R} = \text{OH}$).

In a number of strains an additional characteristic component was detected that showed identical retention times to hydroxyectoine (Fig. 1). In some cocci species hydroxyectoine reached high concentrations and served as the dominant compatible solute. This compound (formerly named Y) has been previously reported and characterized from a halotolerant actinomycete A5-1 (*Nocardioopsis* sp.; Galinski, 1987), a non-halophilic streptomycete (Inbar & Lapidot, 1988) and from salt stressed *Halomonadaceae* (Wohlfarth *et al.*, 1990). The structural formula (Fig. 1) shows that the ectoines differ by a hydroxyl-group in position 3 of the pyrimidine-ring. The steric conformation of the amino and hydroxyl substituents positioned at the two chiral carbon atoms is of the erythro type (E. A. Galinski, unpublished results).

A similar picture emerged from the analysis of halophilic eubacterial organisms obtained from culture collections. Those strains capable of growth on synthetic media were – without exception – producers of organic osmolytes. Among those osmolytes, compounds coeluting with HPLC standards of ectoine and hydroxyectoine were predominant.

To verify our hypothesis that ectoine and hydroxyectoine are the predominant compatible solutes, NMR analyses were necessary. NMR also enabled us to identify solutes of minor concentrations, as well as charged compounds like glutamate that are not detectable by our HPLC system.

Strains containing neither ectoine nor hydroxyectoine are subjects of current investigations.

Identification of compatible solutes by ^{13}C -NMR spectroscopy

Fig. 2 gives three examples of ^{13}C -NMR spectra from chemoheterotrophic *Proteobacteria* grown on saline media. Clearly ectoine is the predominant solute in all cases, a situation which seems to be representative for the vast majority of halophilic/halotolerant Gram-negative chemoheterotrophs, presently available in culture collections. Similarly, our extensive screening revealed that nearly all Gram-negative aerobic halotolerant isolates were ectoine producers.

Fig. 2. Natural abundance ^{13}C -NMR spectra of extracts of two heterotrophic *Proteobacteria*, grown at 30 °C and 10% salts with signals from ectoine (e), glycine betaine (b) and glutamate (gl). (a) *Paracoccus halodenitrificans* grown on glucose-mineral medium, (b) *Vibrio alginolyticus* grown on glucose-mineral medium and (c) *Vibrio alginolyticus* grown on complex medium. Note the occurrence of betaine with growth on complex medium.

Fig. 3 depicts four anoxygenic phototrophic representatives of the *Proteobacteria* exhibiting different salinity ranges for optimal growth and presenting a much wider variability of solutes. *Ectothiorhodospira halochloris* is a typical representative of an extremely halophilic photo-

Fig. 3. Natural abundance ^{13}C -NMR spectra of extracts of four phototrophic *Proteobacteria* grown at various temperatures and salt concentrations with signals from ectoine (e), glycine betaine (b), trehalose (tr), glucosylglycerol (gg), sucrose (sc) and *N*-acetylglutaminylglutamine amide (ga). (a) *Ectothiorhodospira halochloris* (20% salts), (b) *Rhodobacter sulfidophilus* (10% salts), (c) *Rhodospseudomonas marina* (7.5% salts), (d) *Chromatium purpuratum* (7.5% salts). *, Signals of ethanol standard.

troph (growth optimum 20–25% NaCl) which synthesizes glycine betaine as the major compatible solute. Ectoine and trehalose are minor components, present only under certain growth conditions. The moderately halophilic/halotolerant *Rhodobacter sulfidophilus* relies

Fig. 4. Natural abundance ^{13}C -NMR spectra of extracts of four members of *Firmicutes* grown at various temperatures and salt concentrations with signals from ectoine (e), hydroxyectoine (Y), glycine betaine (b), trehalose (tr), glucose (gc), alanine (a) and glutamate (gl). (a) *Actinopolyspora halophila*, 37 °C, glucose-mineral medium, 25% salts; (b) isolate M52, 37 °C, glucose-mineral medium, 15% salts; (c) *Micrococcus halobius*, 30 °C, glucose-mineral medium, 10% salts; and (d) *Micrococcus halophilus*, 30 °C, glucose-mineral medium, 10% salts.

on ectoine as its major osmolyte and minor amounts of glycosylglycerin. *Rhodospseudomonas marina* and *Chromatium purpuratum*, two species of only limited salt tolerance (up to 7.5% NaCl), produce the disaccharides

trehalose and sucrose as their principle solutes. Furthermore, the novel compound *N*-acetyl-glutaminyglutamine amide (Smith & Smith, 1989) is also present in both organisms in minor amounts (E. A. Galinski, unpublished results).

Four spectra of *Firmicutes* showing greater variability are given in Fig. 4. These spectra are distinguished by the presence of ectoine in combination with hydroxyectoine as the predominant osmolytes. The sugars trehalose and glucose, and the amino acids glutamate and alanine were found in minor concentrations.

We have summarized the results of the NMR investigations in Table 1. Data from phototrophic bacteria (Galinski, 1986) are included for comparison. One can conclude that glycine betaine is only synthesized *de novo* by phototrophic bacteria and one extremely halophilic actinomycete *Actinopolyspora halophila* (Fig. 4). This is the only heterotrophic eubacterium known to be capable of betaine synthesis from glucose. In the other heterotrophic eubacteria betaine is only found when they are grown on yeast extract- or choline- and/or betaine-containing media. Therefore, the ectoines (ectoine and hydroxyectoine) clearly represent the predominant solutes within the majority of heterotrophic halophilic strains grown aerobically on glucose-mineral media.

Separation and quantification of organic osmolytes by HPLC

To quantify the constituents of the water soluble fraction of freeze-dried cells, we have used HPLC separation coupled with a refractive index detector. The suitability of this method for the separation of different classes of organic molecules is shown in Fig. 5, which, taking *Micrococcus halobius* as an example, clearly demonstrates the great variability within the solute pattern depending on the carbon source used. Generally, strains able to grow on glycine betaine used this compound as the carbon and energy source as well as the sole compatible solute.

The cellular concentration of osmolytes was calculated on a dry weight basis (weight percent and molar content per g cellular dry weight) and the results from strains obtained from culture collections are presented in Table 2. Of our own isolates only strain M52 is included in Table 2 because of its unusually high content of hydroxyectoine. In general, the other isolates from saline habitats showed similar solute patterns as those strains depicted in Table 2. In addition, Table 2 gives an overview of the growth rates obtained on glucose-mineral media. As high amounts of these solutes are required for further research into possible applications, these data are of importance for the possible selection of producer strains.

Fig. 5. HPLC separation of the compatible solutes of *Micrococcus halobius* on a Nucleosil 5-NH₂ column using 70% acetonitril/water (v/v) as the elution solvent. Variations of solute patterns caused by media composition are shown for *M. halobius* grown on (a) glucose-mineral medium G10, (b) complex medium K10 and (c) betaine-mineral medium B10. Cells were grown in rotary flasks at 30 °C and harvested in the late exponential growth phase. Signals: ectoine (e), hydroxyectoine (Y), glycine betaine (b), trehalose (tr) and glucose (gc). Signals at retention times shorter than 3 min represent solvent peaks. In all three cases the total amount of compatible solutes detected was in the range of 1.1 μmol (mg dry weight)⁻¹ [note different scale in (c)].

Discussion

Although halophilic heterotrophic aerobic eubacteria have been the objects of research for several decades (Rodriguez-Valera, 1988; Vreeland, 1987; Kushner, 1988; Kushner & Kamekura, 1988), their mechanism of osmoadaptation has remained obscure. Neither inorganic ions nor organic molecules were detected in sufficiently high intracellular concentrations to balance the low external water activity. Based on extensive investigations into a large number of organisms isolated from different natural habitats and nearly all heterotrophic halophilic and anoxygenic phototrophic strains available from strain collections (see Tables 1 and 2) we are able to show that the ectoines are the predominant compatible solutes synthesized *de novo* by halophilic aerobic heterotrophic eubacteria, the majority of which belong to the *Proteobacteria* (γ subclass) or the *Firmacutes* (Table 1). In addition, a number of halophilic *Bacillus* strains, which were not included in our study, also seem to be ectoine producers [Müller, 1991, and personal communication (1990)].

The advantage of NMR spectroscopy lies with its unambiguity in the detection of all relevant organic compounds. However, for the purpose of screening and quantification this method is less suitable. We therefore applied an HPLC method (Galinski, 1987; Galinski & Herzog, 1990) which allows the detection and quantification of uncharged solutes from different classes of compounds, such as betaines, carbohydrates, polyols, uncharged amino acids and N-heterocycles. Two limitations of the method have to be considered: first, charged osmolytes are not detected, and second a few substances of similar R_F -value are hard to distinguish unless a second solvent system is applied. Our NMR studies have shown that glutamate and β -glutamate are the only relevant charged osmolytes and they are easily quantified by enzymic tests or amino acid analysis. Nevertheless, only the combination of NMR spectroscopy and chromatography assure a complete qualitative and quantitative characterization of the organic cellular solute content.

In our present work the amount of osmolytes was calculated on the basis of cellular dry weight. This decision was based on the following considerations. It was our aim to present an overview on the occurrence and distribution of compatible solutes in halophilic eubacteria. The cellular dry weight seemed to be a suitable reference as it is easily measured and reproduced. For biotechnological purposes, such as comparison of productivity, the amount of solutes per g dry weight can be easily expressed in terms of yield coefficients. On the other hand, volume determinations, which allow the estimation of intracellular concentrations, are very time-consuming because the determined

cell volume is affected by the method applied, salt concentration, temperature or growth phase (Kubitschek, 1987; Stock *et al.*, 1977). A simple calculation based upon a dry weight/cytoplasmic water ratio of 1:0.8 (or higher) will provide an estimate for the intracellular concentration of compatible solutes: 1 mol (kg cytoplasmic water)⁻¹ minimum.

Compatible solute studies performed on marine and halophilic cyanobacteria (Mackay *et al.*, 1984; Reed *et al.*, 1986) have led to the proposal that the type of osmolyte employed reflects the salinity range suitable for the organism. Thus, the occurrence of betaine seemed to be typical for organisms of high salt tolerance. Glucosylglycerol reflected an intermediate salinity range and the use of sugars as osmolytes was mostly confined to fresh water and marine species.

This general principle also seems to hold true for anoxygenic phototrophic bacteria, but further compounds which have so far not been detected in cyanobacteria must also be considered. Examples are the glutamine derivative *N*-acetyl-glutaminylglutamine amide (Fig. 3), which has also been reported from the non-halophilic *Rhizobium meliloti* (Smith & Smith, 1989) under salt stress and carbamoyl glutaminamide in *E. marismortui* (Galinski & Oren, 1991). The most remarkable difference, however, is the ability of anoxygenic phototrophic bacteria to synthesize and accumulate ectoine, a compound which has not been found in cyanobacteria in molar concentrations. This solute was originally detected in and, hence, named after the genus *Ectothiorhodospira* (Galinski *et al.*, 1985), but has now proved to be the predominant compatible solute of heterotrophic halophilic eubacteria.

With the exception of *A. halophila*, heterotrophic halophilic eubacteria are apparently unable to synthesize glycine betaine *de novo*, whereas in most cases they showed high rates of synthesis of ectoines. Surprisingly, these tetrahydropyrimidines were also identified in a non-halophilic *Streptomyces parvulus* strain (Inbar & Lapidot, 1988) and it seems possible that biosynthesis of these compounds is more common in non-halophilic eubacteria than is currently believed. The biosynthetic pathway of ectoine branches off the metabolic route forming amino acids of the aspartate family (Peters *et al.*, 1990) and seems to be less complex than betaine synthesis (Trüper & Galinski, 1990).

According to the work presented here, the importance of betaine as an osmolyte has to be looked at more distinctively than before (Imhoff, 1986; Imhoff & Rodriguez-Valera, 1984). Only in (extremely) halophilic phototrophic bacteria does betaine seem to be the main compatible solute, whereas *de novo* synthesis in chemo-heterotrophic eubacteria seems to be the exception.

The data available at present, including the results obtained for cyanobacteria lead us to propose the following principles.

(1) Eubacteria capable of haloadaption that gain energy from photosynthesis or oxygen respiration are able to accumulate and/or produce compatible solutes in molar concentrations.

(2) Betaine is very common as a compatible solute among phototrophic eubacteria, whereas, of all heterotrophic bacteria investigated, only *Actinopolyspora halophila* and a related isolate were capable of betaine synthesis from simple carbon sources like glucose.

(3) All eubacteria tested were able to accumulate betaine from the medium or use possible precursors like choline for betaine synthesis.

(4) Ectoine, originally detected in minor concentrations in anoxygenic phototrophic bacteria, is the predominant compatible solute of heterotrophic halophilic aerobic bacteria grown on glucose-mineral media.

(5) Hydroxyectoine, found in heterotrophic eubacteria, seems to be more common and present in higher concentrations among Gram-positive representatives.

(6) Solutes occurring in minor concentrations (< 1 M) are glutamate, β -glutamate, alanine, sucrose, trehalose, glucose, glycosylglycerol, *N*-acetyl-glutaminylglutamine amide, carbamoylglutamine amide and other yet unidentified compounds.

(7) More than one osmolyte can contribute to the cellular solute pool. The proportion of solutes varies with respect to media composition and growth conditions. (Table 2; Wohlfarth *et al.*, 1990).

(8) Some strains were found to accumulate compatible solutes but were not capable of ectoine or betaine synthesis (Severin *et al.*, 1989).

We are well aware of the fact that the experimental design for our, and possibly other screening procedures influences the type of organisms selected. Standard enrichment media usually contain high concentrations of carbon source and therefore select for organisms of high growth rate and good colony forming abilities. Design of more unusual screening techniques such as, for example, continuous culture for the enrichment of strains with high substrate affinity may well present us with a new range of organisms which do not conform with the discussed concept.

The typical sampling sites for this study, however, were solar salterns and hypersaline soils which are characterized by a high organic carbon content. Therefore, we believe that our screening results reflect a fairly true picture of the microbial populations of these biotops.

The stabilizing effects of osmolytes such as glycerol and sugars on enzymes and whole cells was well known

long before the term compatible solute was introduced. Subsequently, enzyme protection has also been demonstrated for glycine betaine (Pollard & Wyn Jones, 1979) and, more recently, for the novel ectoines, which have attracted considerable attention due to their remarkable protection against heat, freezing and drying (Lippert & Galinski, 1992). It is therefore justified to assume that compatible solutes in general have protective properties and may well find an application in enzyme technology. Ectoines, especially hydroxyectoine, which has two asymmetric carbon atoms, are difficult and costly to synthesize chemically. Biotechnological production is presently the only means to supply material for further experimental research. Our screening programme, therefore, also served the purpose of providing potent producer strains for these interesting compounds.

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References

- AUSTIN, B. (1989). Novel pharmaceutical compounds from marine bacteria. *Journal of Applied Bacteriology* **67**, 461–470.
- AVRON, M. (1986). The osmotic components of halotolerant algae. *Trends in Biochemical Sciences* **11**, 5–6.
- BEN-AMOTZ, A. & AVRON, M. (1983). Accumulation of metabolites by halotolerant algae and its industrial potential. *Annual Reviews in Microbiology* **37**, 95–119.
- BROWN, A. D. (1976). Microbial water stress. *Bacteriological Reviews* **40**, 803–846.
- CAUMETTE, P., BAULAIGUE, R. & MATHERON, R. (1988). Characterization of *Chromatium salexigens* sp. nov., a halophilic Chromatiaceae isolated from mediterranean salinas. *Systematic and Applied Microbiology* **10**, 284–292.
- CAUMETTE, P., BAULAIGUE, R. & MATHERON, R. (1991). *Thiocapsa halophila* sp. nov., a new halophilic purple sulfur bacterium. *Archives of Microbiology* **155**, 170–176.
- FENDRICH, C. (1988). *Halovibrio variabilis* gen. nov. spec., *Pseudomonas halophila* sp. nov. and a new halophilic aerobic coccoid eubacterium from Great Salt Lake, Utah, USA. *Systematic and Applied Microbiology* **11**, 36–43.
- GALINSKI, E. A. (1986). *Salzadaptation durch kompatible Solute bei halophilen phototrophen Bakterien*. PhD Thesis. University of Bonn.
- GALINSKI, E. A. (1987). Halo-fermentation, a novel low water process for the production of organic chemicals and enzyme protective agents. In *Bioreactors and Biotransformations*, pp. 201–212. Edited by G. W. Moody & P. B. Baker. London: Elsevier.
- GALINSKI, E. A. & TRÜPER, H. G. (1982). Betaine, a compatible solute in the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochloris*. *FEMS Microbiology Letters* **13**, 357–360.
- GALINSKI, E. A. & HERZOG, R. M. (1990). The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). *Archives of Microbiology* **153**, 607–613.
- GALINSKI, E. A. & OREN, A. (1991). Isolation and structure determination of a novel compatible solute from the moderately halophilic purple sulfur bacterium *Ectothiorhodospira marismortui*. *European Journal of Biochemistry* **198**, 593–598.
- GALINSKI, E. A. & TINDALL, B. J. (1991). Biotechnological prospects of halophilic and halotolerant microorganisms. In *Molecular Biology and Biotechnology of Extremophiles*, pp. 76–114. Edited by R. A. Herbert & R. J. Sharp. Glasgow: Blackie.
- GALINSKI, E. A., PFEIFFER, H. P. & TRÜPER, H. G. (1985). 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinocarboxylic acid – a novel cyclic amino acid from halophilic phototrophic bacteria of the genus *Ectothiorhodospira*. *European Journal of Biochemistry* **149**, 135–139.
- GROTHE, S., KROGSrud, R. L., MCCELLAN, D. J., MILNER, J. L. & WOOD, J. M. (1986). Proline transport and osmotic stress response in *Escherichia coli* K-12. *Journal of Bacteriology* **166**, 253–259.
- IMHOFF, J. F. (1986). Osmoregulation and compatible solutes in eubacteria. *FEMS Microbiology Reviews* **39**, 57–66.
- IMHOFF, J. F. & TRÜPER, H. G. (1977). *Ectothiorhodospira halochloris* sp. nov., a new extremely halophilic phototrophic bacterium containing bacteriochlorophyll b. *Archives of Microbiology* **114**, 115–121.
- IMHOFF, J. F. & RODRIGUEZ-VALERA, F. (1984). Betaine is the main compatible solute of halophilic eubacteria. *Journal of Bacteriology* **160**, 478–479.
- INBAR, L. & LAPIDOT, A. (1988). The structure and biosynthesis of new tetrahydropyrimidine derivatives in actinomycin D producer *Streptomyces parvulus*. *Journal of Biological Chemistry* **263**, 16014–16022.
- JOVALL, P.-A., TUNBLAD-JOHANSSON, I. & ADLER, L. (1990). ¹³C NMR analysis of production and accumulation of osmoregulatory metabolites in the salt-tolerant yeast *Debaryomyces hansenii*. *Archives of Microbiology* **154**, 209–214.
- KANEMASA, Y., YOSHIOKA, T. & HAYASHI, H. (1972). Alteration of the phospholipid composition of *Staphylococcus aureus* cultured in medium containing NaCl. *Biochimica et Biophysica Acta* **280**, 444–450.
- KOMARATAT, P. & KATES, M. (1975). The lipid composition of a halotolerant species of *Staphylococcus epidermidis*. *Biochimica et Biophysica Acta* **398**, 464–484.
- KUBITSCHKE, H. E. (1987). Buoyant density variation during the cell cycle in microorganisms. *CRC Critical Reviews in Microbiology* **14**, 73–97.
- KUSHNER, D. J. (1988). What is the 'true' internal environment of halophilic and other bacteria? *Canadian Journal of Microbiology* **34**, 482–486.
- KUSHNER, D. J. & KAMEKURA, M. (1988). Physiology of halophilic eubacteria. In *Halophilic Bacteria*, vol. 1, pp. 109–138. Edited by F. Rodriguez-Valera. Boca Raton, Florida: CRC Press.
- LAI, M.-C., SOWERS, K. R., ROBERTSON, D. E., ROBERTS, M. F. & GUNSALUS, R. P. (1991). Distribution of compatible solutes in the halophilic methanogenic archaeobacteria. *Journal of Bacteriology* **173**, 5352–5358.
- LARSEN, P. I., SYDNES, L. K., LANDFALD, B. & STROM, A. R. (1987). Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. *Archives of Microbiology* **147**, 1–7.
- LE RUDULIER, D. & BERNARD, T. (1986). Salt tolerance in *Rhizobium*: a possible role for betaines. *FEMS Microbiology Reviews* **39**, 67–72.
- LIPPERT, K. & GALINSKI, E. A. (1992). Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. *Applied Microbiology and Biotechnology* **37**, 61–65.
- MACKAY, M. A., NORTON, R. S. & BOROWITZKA, L. J. (1984). Organic osmoregulatory solutes in cyanobacteria. *Journal of General Microbiology* **130**, 2177–2191.
- MÜLLER, E. (1991). *Kompatible Solute und Prolingewinnung bei halophilen und halotoleranten Bacilli*. PhD Thesis. University of Bonn.
- OKAMI, Y. (1986). Marine microorganisms as a source of bioactive agents. *Microbiological Ecology* **12**, 65–79.
- OREN, A. (1985). Intracellular salt concentrations of the anaerobic halophilic eubacteria *Haloanaerobium praevalens* and *Halobacteroides halobius*. *Canadian Journal of Microbiology* **32**, 4–9.
- OREN, A., KESSEL, M. & STACHEBRANDT, E. (1989). *Ectothiorhodospira marismortui* sp. nov., an obligately anaerobic, moderately halophilic

- purple sulfur bacterium from a hypersaline sulfur spring on the shore of the Dead Sea. *Archives of Microbiology* **151**, 524–529.
- PETERS, P., GALINSKI, E. A. & TRÜPER, H. G. (1990). The biosynthesis of ectoine. *FEMS Microbiology Letters* **71**, 157–162.
- POLLARD, A. & WYN JONES, R. G. (1979). Enzyme activities in concentrated solutions of glycine betaine and other solutes. *Planta* **144**, 291–298.
- REED, R. H., BOROWITZKA, L. J., MACKAY, M. A., CHUDEK, J. A., FOSTER, R., WARR, S. C. R., MOORE, D. J. & STEWART, W. D. P. (1986). Organic solute accumulation in osmotically stressed cyanobacteria. *FEMS Microbiology Reviews* **39**, 51–56.
- REGEV, R., PERI, I., GILBOA, H. & AVI-DOR, Y. (1990). ¹³C NMR study of the interrelation between synthesis and uptake of compatible solutes in two moderately halophilic eubacteria. *Archives of Biochemistry and Biophysics* **277**, 106–112.
- RENGPIPAT, S., LOWE, S. E. & ZEIKUS, J. G. (1988). Effect of extreme salt concentrations on the physiology and biochemistry of *Halobacteroides acetothylus*. *Journal of Bacteriology* **170**, 3065–3071.
- ROBERTSON, D. E., ROBERTS, M. F., BELAY, N., STETTER, K. O. & BOONE, D. R. (1990a). Occurrence of β -glutamate, a novel osmolyte, in marine methanogenic bacteria. *Applied and Environmental Microbiology* **56**, 1504–1508.
- ROBERTSON, D. E., NOLL, D., ROBERTS, M. F., MENAIA, J. A. G. F. & BOONE, D. R. (1990b). Detection of the osmoregulator betaine in methanogens. *Applied and Environmental Microbiology* **56**, 563–565.
- RODRIGUEZ-VALERA, F. (1988). *Halophilic Bacteria*, vol. 1. Boca Raton, Florida: CRC Press.
- SEVERIN, J., WOHLFARTH, A. & GALINSKI, E. A. (1989). Evidence for new compatible solutes from halotolerant bacteria not capable of ectoine synthesis. *Proceedings of the FEMS-NATO workshop on General and Applied Aspects of Halophilic Microorganisms* 17–22 September 1989, Alicante, Spain.
- SMITH, L. T. & SMITH, G. M. (1989). An osmoregulated dipeptide in stressed *Rhizobium meliloti*. *Journal of Bacteriology* **171**, 4714–4717.
- SMITH, L. T., POCARD, J. A., BERNARD, T. & LE RUDULIER, D. (1988). Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. *Journal of Bacteriology* **170**, 3142–3149.
- STALEY, J. T. & STANLEY, P. M. (1986). Potential commercial applications in aquatic microbiology. *Microbiological Ecology* **12**, 79–100.
- STOCK, J. B., RAUCH, B. & ROSEMAN, S. (1977). Periplasmic volume changes in *Salmonella typhimurium* and *Escherichia coli*. *Journal of Biological Chemistry* **252**, 7850–7861.
- TEMPEST, D. W., MEERS, J. L. & BROWN, C. M. (1970). Influence of environment on the content and composition of microbial free amino acid pools. *Journal of General Microbiology* **64**, 171–185.
- TRÜPER, H. G. & GALINSKI, E. A. (1986). Concentrated brines as habitats for micro-organisms. *Experientia* **42**, 1182–1187.
- TRÜPER, H. G. & GALINSKI, E. A. (1990). Biosynthesis and fate of compatible solutes in extremely halophilic phototrophic eubacteria. *FEMS Microbiology Reviews* **75**, 247–254.
- TRÜPER, H. G., SEVERIN, J., WOHLFARTH, A., MÜLLER, E. & GALINSKI, E. A. (1991). Halophily, taxonomy, phylogeny and nomenclature. In *General and Applied Aspects of Halophilic Microorganisms*. NATO ASI Series A: Life Sciences vol. 201, pp. 3–7. Edited by F. Rodriguez-Valera. New York: Plenum Press.
- VAN ECK, J. H., PRIOR, B. A. & BRANDT, E. V. (1989). Accumulation of polyhydroxy alcohols by *Hansenula anomala* in response to water stress. *Journal of General Microbiology* **135**, 3505–3513.
- VENTOSA, A., GUTIERREZ, M. C., GARCIA, M. T. & RUIZ-BERRAQUERO, F. (1989). Classification of '*Chromobacterium marismortui*' in a new genus *Chromohalobacter* gen. nov., as *Chromohalobacter marismortui* comb. nov., nom. rev. *International Journal of Systematic Bacteriology* **39**, 382–386.
- VREELAND, R. H. (1987). Mechanisms of halotolerance in micro-organisms. *CRC Critical Reviews in Microbiology* **14**, 311–356.
- WEGMANN, K. (1986). Osmoregulation in eucaryotic algae. *FEMS Microbiology Reviews* **39**, 37–43.
- WHATMORE, A. M., CHUDEK, J. A. & REED, R. H. (1990). The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. *Journal of General Microbiology* **136**, 2527–2535.
- WOESE, C. R. (1987). Bacterial evolution. *Microbiology Reviews* **51**, 222–271.
- WOHLFARTH, A., SEVERIN, J. & GALINSKI, E. A. (1989). Screening for compatible solutes of halophilic eubacteria. In *Microbiology of Extreme Environments and its Potential for Biotechnology*, p. 421. Proceedings of the Federation of European Microbiological Societies Symposium held in Troia, Portugal, 18–23 September 1988. Edited by M. S. da Costa, J. C. Duarte & R. A. D. Williams. London: Elsevier Science Publishers.
- WOHLFARTH, A., SEVERIN, J. & GALINSKI, E. A. (1990). The spectrum of compatible solutes in heterotrophic halophilic eubacteria of the family *Halomonadaceae*. *Journal of General Microbiology* **136**, 705–712.