Extremophiles (2002) 6:491–498
Digital Object Identifier (DOI) 10.1007/s00792-002-0286-3

ORIGINAL PAPER

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Intracellular ion and organic solute concentrations of the extremely halophilic bacterium *Salinibacter ruber*

Received: January 17, 2002 / Accepted: June 26, 2002 / Published online: August 24, 2002

Abstract *Salinibacter ruber* is a red obligatory aerobic chemoorganotrophic extremely halophilic Bacterium, related to the order Cytophagales. It was isolated from saltern crystallizer ponds, and requires at least 150 g l^{-1} salt for growth. The cells have an extremely high potassium content, the ratio K^+/protein being in the same range as in halophilic Archaea of the order Halobacteriales. X-ray microanalysis in the electron microscope of cells grown in medium of 250 g l^{-1} salt confirmed the high intracellular K^+ concentrations, and showed intracellular chloride to be about as high as the cation concentrations within the cells. A search for intracellular organic osmotic solutes, using ^{13}C-NMR and HPLC techniques, showed glutamate, glycine betaine, and *N*-α-acetyllysine to be present in low concentrations only, contributing very little to the overall osmotic balance. The results presented suggest that the extremely halophilic Bacterium *Salinibacter* uses a similar mode of haloadaptation to that of the Archaea of the order Halobacteriales, and does not accumulate organic osmotic solutes such as are used by all other known halophilic and halotolerant aerobic Bacteria.

Key words Compatible solutes · Halophilic · Intracellular ion concentrations · *Salinibacter* · X-ray microanalysis

Communicated by W. Grant

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Introduction

Salinibacter ruber is a red, obligatory aerobic, chemoorganotrophic, extremely halophilic Bacterium. The species was recently isolated from saltern ponds in Spain (Antón et al. 2002). Phylogenetically it is related to the order Cytophagales. Its closest known relative is the marine thermophilic red bacterium *Rhodothermus marinus*, found in submarine hot springs (Antón et al. 2000, 2002). *Salinibacter* is often found in high numbers in saltern crystallizer ponds, and may represent up to 5%–25% of the total prokaryote community in such environments (Antón et al. 2000; Oren and Rodríguez-Valera 2001).

S. ruber requires at least 150 g l^{-1} salt for growth, and grows optimally at salt concentrations between 200 and 250 g l^{-1}. Therefore it is as halophilic and salt-dependent as the most salt-dependent of the halophilic Archaea such as *Halobacterium* or *Haloarcula* species. Another property that *S. ruber* shares with the members of the Halobacteriales is a high content of acidic amino acids in its proteins and the resistance toward, and in some cases the requirement for, salt of its intracellular enzymes (Oren and Mana 2002). The question to be asked is therefore whether *S. ruber* indeed uses a haloadaptation strategy similar to that of the aerobic halophilic Archaea.

All the halophilic and halotolerant aerobic Bacteria that have been characterized thus far produce and/or accumulate organic "compatible" solutes such as ectoine, glycine betaine, and others, to provide the necessary osmotic balance. They actively exclude salt from their cytoplasm to a large extent. Synthesis and degradation of compatible solutes can be regulated according to the extracellular salt concentration, and this strategy generally enables a considerable degree of adaptability to changes in the salinity of the medium (Oren 1999; Ventosa et al. 1998). The aerobic halophilic Archaea, on the other hand, accumulate KCl in molar concentrations (Christian and Waltho 1962; Lanyi 1974). This strategy requires far-reaching adaptations in order for all intracellular processes to be functional at high salt concentrations. Proteins of the Halobacteriales are typically rich in acidic amino acids, depleted of basic amino acids, and

relatively poor in hydrophobic amino acids. Such proteins generally require the presence of high salt concentrations for stability and activity (Dennis and Shimmin 1997; Lanyi 1974). Accordingly, the microorganisms that harbor these are unable to adapt to life below a (generally very high) minimal salt concentration. A similar strategy of adaptation to high salt was found in the obligatory anaerobic Bacteria of the order Halanaerobiales, phylogenetically affiliated with the low G+C branch of the Firmicutes (Oren 1986; Oren et al. 1997; Rengpipat et al. 1988).

In this paper we provide information on the intracellular ion content of *S. ruber* cells. We provide evidence of the presence of extremely high KCl concentrations in the cytoplasm. This, together with the apparent absence of significant concentrations of organic osmotic solutes and the earlier documented presence of the typical "halophilic" traits in the amino acid composition of the cellular proteins (Oren and Mana 2002), shows that this novel group of halophilic Bacteria uses KCl to provide osmotic balance, similar to the red halophilic Archaea of the order Halobacteriales.

Materials and methods

Bacterial and archaeal strains and culture conditions

Salinibacter ruber strain M31 (DSM 13855^T) (isolated from a saltern on the Spanish island of Mallorca), as well as strains Pola-13 and Pola-18, isolated from a crystallizer pond of the salterns of Santa Pola near Alicante, Spain (Antón et al. 2002), were routinely grown in medium containing (all concentrations in g l^{-1}): NaCl, 195; $MgSO_4.7H_2O$, 25; $MgCl_2.6H_2O$, 16.3; $CaCl_2.2H_2O$, 1.25; KCl, 5.0; $NaHCO_3$, 0.25; NaBr, 0.625; yeast extract, 1.0; and pH 7.0. In some of the experiments designed to assess intracellular concentrations of organic osmotic solutes, the yeast extract was replaced by 2 g l^{-1} casamino acids, 0.2 g l^{-1} NH_4Cl, 0.1 g l^{-1} KH_2PO_4, and 2 ml l^{-1} of a vitamin solution containing (per liter): biotin, 10 mg; pyridoxine.HCl, 50 mg; riboflavin, 25 mg; Ca-pantothenate, 25 mg; vitamin B_{12}, 0.05 mg; folic acid, 10 mg; thiamine.HCl, 25 mg; nicotinic acid, 25 mg; and p-aminobenzoic acid, 5 mg. For elemental analysis in the electron microscope the NaCl concentration in the standard medium was increased to 225 g l^{-1}, the concentrations of $MgSO_4.7H_2O$ and $MgCl_2.6H_2O$ were decreased to 2.5 g l^{-1}, $CaCl_2.2H_2O$ was added at 0.25 g l^{-1}, and NaBr was omitted from the medium.

Halobacterium salinarum R1 was grown in medium containing (all concentrations in g l^{-1}): NaCl, 250; KCl, 5.0; $MgCl_2.6H_2O$, 5.0; NH_4Cl, 5.0; and yeast extract, 10; pH 7.0. The medium for *Haloarcula marismortui* ATCC 43049^T was composed of NaCl, 206; $MgSO_4.7H_2O$, 36; KCl, 0.37; $CaCl_2.2H_2O$, 0.5; $MnCl_2$, 0.013; and yeast extract, 5.0; pH 7.0. *Halomonas elongata* (ATCC 33173^T) was grown either in complex medium (NaCl, 100; KCl, 1.0; $MgSO_4.7H_2O$, 10; yeast extract, 20; Tris, 15; pH 7.5) or in minimal medium (NaCl, 100 or 200 as indicated; $MgSO_4.7H_2O$, 2.8; NH_4Cl, 4.5; $FeSO_4.7H_2O$, 0.01; Tris, 1.5; KH_2PO_4, 1.1; and glucose, 2.0 (the last two components added to the autoclaved medium from separately sterilized concentrated solutions); pH 7.5. *Salinivibrio costicola* (ATCC 33508^T) was grown in medium that contained NaCl, 200; KCl, 1.0; $MgCl_2.6H_2O$, 10; and nutrient broth (Difco), 8.0; pH 7.0.

All the above-mentioned bacteria were grown on a rotary shaker (200 rpm) at 35°C in 100-ml portions in 250-ml Erlenmeyer flasks or in 1-l portions in 2-l Erlenmeyer flasks. Mid-exponential growth phase cells were used in all experiments. For electron microscopical elemental analyses, requiring cultures of low cell density, early exponential phase cultures were examined.

Halanaerobium praevalens DSM 2228^T was grown at 35°C in medium containing (g l^{-1}): NaCl, 130; $MgSO_4.7H_2O$, 8.8; KCl, 1; yeast extract, 10; tryptone, 10; cysteine.HCl, 0.5; and glucose, 2.5; pH 7.8. Anaerobic medium was prepared by boiling under nitrogen. Glucose was added to the autoclaved medium from a separately sterilized anaerobic solution, and the pH was adjusted with an anoxic solution of 1 M NaOH. The medium was dispensed under nitrogen into 50-ml portions in 129-ml serum bottles provided with butyl rubber stoppers and aluminum crimp seals (Oren 1986).

Quantitation of cell-associated sodium and potassium by flame photometry

Cells were collected by centrifugation (1 min, 10,000 *g*) in 1.5-ml plastic centrifuge tubes. Cell pellets, containing between 0.16 and 0.68 mg protein, as assessed using the Lowry procedure (Lowry et al. 1951), were washed once with a salt solution containing the inorganic salts of the respective growth medium from which potassium was omitted. Pellets were then extracted with 0.2 ml 10% perchloric acid for 2 h, and extracts were diluted with distilled water (for potassium assays to K^+ concentrations between 0.2 and 0.8 mM; for sodium assays to Na^+ concentrations between 0.1 and 0.5 mM), whereafter the concentrations of K^+ and Na^+ were determined by flame photometry. All assays were performed at least in triplicate.

Na^+ and K^+ analysis in cell pellets

Intracellular Na^+ and K^+ concentrations of *S. ruber* were estimated by analyzing extracts of cell pellets, using ^3H_2O as a marker for the total water space and blue dextran for the extracellular space, in a procedure resembling the protocols used by Oren (1986) and Shindler et al. (1977). Parallel experiments were done with *Halobacterium salinarum*. Mid-exponential growth phase cells, grown in the standard medium as described above, were collected by centrifugation (10 min, 3,400 *g*) and resuspended to a density of approximately 40–80 mg wet pellet weight ml^{-1} in fresh growth medium amended with 10 mg ml^{-1} blue dextran (Sigma), dissolved by gentle heating. Portions of 1 ml of cell suspension were added to 1.5-ml plastic centrifuge tubes.

^3H_2O (Amersham) (0.1 µCi in 20 µl) was added. After 5 min incubation at room temperature, the contents of the tube were centrifuged for 2 min at 12,000 *g*. A portion of 50 µl of the supernatant was mixed with 0.95 ml 10% perchloric acid, the remainder of the supernatant was removed by means of a Pasteur pipette, and the pellet was resuspended in 0.95 ml 10% perchloric acid. After 2 h extraction at room temperature the tubes were centrifuged again. Both supernatant fractions were analyzed for Na^+ and K^+ (by flame photometry), blue dextran content (by absorbance at 600 nm) and ^3H radioactivity (by liquid scintillation counting of 50 µl portions with 2.5 ml of Zinsser Quicksafe A scintillation cocktail). Calculations of intracellular Na^+ and K^+ concentrations were based on the assumption that blue dextran did not penetrate into the cells and was not absorbed on the surface of the cells to any significant extent, and that ^3H_2O had reached complete equilibration with the cell contents.

Elemental analyses of single bacterial cells in the electron microscope

Early exponential phase cells were collected by centrifugation in a Beckman model L8–70M preparative ultracentrifuge, using a SW41 swing-out rotor at 17,000 *g* for 10 min at 20°C on aluminum grids (100-mesh, Agar Scientific) supported with a carbon-coated formvar film, and then air-dried. Cells were viewed and analyzed for light elements in a Philips CM200 electron microscope. The sample grids were mounted between beryllium rings in a Compustage low background double tilt holder (Type PW6595/15). The microscope was operated in scanning mode at a tilt angle of 31°, an acceleration voltage of 80 kV, a magnification between 5,000× and 10,000×, a spot size of 14 nm (spot size 3), and an accumulation time (live time) of 30 s. The X-ray spectrum was recorded from an area that tightly circumscribes the specimen (Norland et al. 1995). The light element detection system consisted of EDAX detector DX-4, combined with SIS Soft Imaging Software. For each preparation, between 20 and 49 cells were analyzed. To quantify the contribution of the supporting film, the spectra of particle-free areas of identical size and shape adjacent to each cell were recorded. No salt crystals were observed intracellularly, attached to the cells, or in the area between the cells. Calibration constants for the different elements were determined according to Norland et al. (1995).

Extraction of compatible solutes

Lyophilized bacterial cell mass was extracted following a modification of the universal fast extraction method described by Galinski and Herzog (1990). For natural abundance ^{13}C-NMR analysis, a minimum of 1 g dried bacterial cell mass was used, and the aqueous solute-containing phase was concentrated by evaporating the solvent at reduced pressure and dissolving the residue in 1 ml D_2O.

Natural-abundance ^{13}C-NMR spectroscopy

Samples were recorded in the pulsed Fourier transform mode on a Varian (model ARX300) Fourier transform spectrometer operating at 75.48 MHz (^{13}C) and at 300 MHz for the proton decoupling channel relative to trimethylsilylpropionate (sodium salt).

High performance liquid chromatography (HPLC) of compatible solutes

Compatible solutes belonging to the classes polyols, sugars, and zwitterionic amino acid derivatives were analyzed according to the method of Galinski and Herzog (1990). Detection was carried out using an isocratic HPLC-system from Thermo Separation Products (CA), consisting of an isocratic pump (P100), a 3-µm Grom-sil 100 Amino-1PR modified reversed phase column (Grom Herrenberg-Kayh), UV-Detector (UV1000) operated at 210 nm, and a Shodex refractive index detector (Showa Denko KK, Tokyo), model RI71. The mobile phase consisted of 80% (v/v) acetonitrile at a flow rate of 1 ml min^{-1}. For peak integration Chrom Quest software was used.

High performance liquid chromatography (HPLC) of amino-reactive compounds

Amino acids and other amino-reactive compounds in the extracts were determined using 9-fluorenylmethoxycarbonyl chloride (FMOC) HPLC using a modification of the method of Kunte et al. (1993). The HPLC-system (Thermo Separation Products, CA) consisted of a gradient delivery pump (P2000), a 4-µm Superspher 60 RP-8 column 125 × 4 mm (Grom, Herrenberg-Kayh), a fluorescence-detector (model FL3000) set at 254 nm for excitation and 316 nm as emission wavelength, and computer-based integration software. The mobile phase consisted of solvent A [20% (v/v) acetonitrile and 0.5% tetrahydrofuran in 50 mM sodium acetate buffer, pH 4.2] and solvent B (80% acetonitrile in 50 mM sodium acetate buffer, pH 4.2). Chromatography was performed at 45°C and a flow rate of 1.25 ml min^{-1} using the following time profile: 0% B (0 min), 9% B (15 min), 30% B (30 min), 60% B (40 min), 100% B (42 min), 100% B (47 min), 0% B (49 min), and 0% B (51 min).

Results

Intracellular ion concentrations in *Salinibacter ruber*

S. ruber cells contain extremely high concentrations of K^+ ions in their cytoplasm. Table 1 presents a comparison of the potassium content of two *S. ruber* strains, calculated per unit of cell protein, with that of other halophilic microorganisms. Values of 15.2 and 11.4 µmol K^+ mg $protein^{-1}$, as

Table 1. Potassium content per unit of cellular protein in *Salinibacter* isolates, as compared with other halophilic and halotolerant bacteria

Species	Growth medium and conditions	K^+/protein (μmol mg^{-1})
Salinibacter ruber strain M31 (DSM 13855^T)	196 g/l NaCl, total salt concentration 250 g/l; 0.1% yeast extract; cells washed in 25% NaCl + 2% $MgCl_2.6H_2O$, pH 7	11.4 ± 1.1 (n = 6)
Salinibacter ruber strain Pola-18	200 g/l NaCl, total salt concentration 210 g/l; cells washed in 25% NaCl + 2% $MgCl_2.6H_2O$, pH 7	15.2 ± 0.6 (n = 3)
Haloarcula marismortui ATCC 43049^T	206 g/l NaCl, total salt concentration 225 g/l; 0.5% yeast extract; cells washed in 25% NaCl + 2% $MgCl_2.6H_2O$, pH 7	13.2 ± 0.4 (n = 3)
Halobacterium salinarum strain R1	250 g/l NaCl, total salt concentration 263 g/l; 1% yeast extract; cells washed in 25% NaCl + 2% $MgCl_2.6H_2O$, pH 7	12.0 ± 0.7 (n = 7)
Halanaerobium praevalens DSM 2228^T	130 g/l NaCl, total salt concentration 135 g/l; 1% yeast extract + 1% tryptone; anaerobic conditions; cells washed in 15% NaCl, pH 7	6.3 ± 0.3 (n = 3)
Halomonas elongata ATCC 33173^T	100 g/l NaCl, total salt concentration 106 g/l; 1% yeast extract; cells washed in 10% NaCl, pH 7	1.1 ± 0.2 (n = 3)
Halomonas elongata ATCC 33173^T	100 g/l NaCl, total salt concentration 106 g/l; defined medium with glucose as sole carbon source; cells washed in 10% NaCl, pH 7	2.2 ± 0.1 (n = 3)

Exponentially growing cells were harvested by centrifugation, washed, and recentrifuged. Pellets were extracted in 10% perchloric acid for flame-photometric determination of potassium ions, or assayed for protein according to Lowry et al. (1951). Mean values and standard deviations were based on 3–4 independent determinations

measured in *S. ruber*, are in the same order of magnitude as those found in *Halobacterium salinarum* and *Haloarcula marismortui*, organisms that use KCl to osmotically balance the high NaCl concentration in the surrounding medium. Much lower values (1.1–2.2 μmol K^+ mg $protein^{-1}$, depending on the growth conditions) were measured in *Halomonas elongata*, a Bacterium that accumulates organic osmotic solutes by synthesis and/or transport from the medium. Intermediate values were found in the anaerobic Bacterium *Halanaerobium praevalens*, a representative of the Halanaerobiales, a group earlier shown to use KCl to provide osmotic balance (Oren 1986; Oren et al. 1997). Protein-related values of cytoplasmic volume in halophilic prokaryotes generally vary between 2 and 5 μl mg $protein^{-1}$, and for *Halobacterium salinarum* values of 2.5–2.75 μl mg $protein^{-1}$ have been reported (Imhoff and Riedl 1989 and references therein). When assuming a value of 2.75 μl mg $protein^{-1}$, both for the halophilic Archaea tested and for *S. ruber*, the estimated intracellular K^+ concentrations would be 4.4 and 4.8 M, respectively, in *Halobacterium salinarum* and *Haloarcula marismortui* (values close to those reported), and 4.1–5.5 M in *S. ruber*.

Attempts to estimate cytoplasmic K^+ concentrations in cell pellets yielded values of 1.21 ± 0.06 M (n = 5), 0.89 ± 0.08 M (n = 4), and 0.69 ± 0.05 M (n = 6) for *S. ruber* (results from three independent experiments), and 2.93 ± 0.14 M (n = 2) for *Halobacterium*. In these experiments the apparent intracellular volume varied between 46.8% and 59.4% of the total pellet volume. Apparent sodium concentrations thus measured were exceedingly high: in one experiment the calculated Na^+ concentration in *Salinibacter* cells was 3.50 ± 0.72 M (n = 5), while in the other experiments the apparent intracellular Na^+ concentration exceeded that of the extracellular medium. This may point both to problems with the exact estimation of intracellular and extracellular volumes, and to the possible leaking of ions during handling of the cells.

The presence of high potassium concentrations inside *S. ruber* cells, accompanied by high concentrations of chloride as the counterion, was confirmed during measurements of the elemental composition of single cells in the X-ray microprobe in the electron microscope (Table 2). Apparent concentrations of K^+, Na^+, Mg^{2+} and Cl^- in *S. ruber* grown in medium containing 3.3 M NaCl were 0.59, 0.38, 0.29, and 0.9 M, respectively. For comparison, the corresponding values for *Halobacterium salinarum* grown in medium of a comparable salinity were 0.93, 0.22, 0.03, and 1.14 M. The Bacteria *Halomonas elongata* and *Salinivibrio costicola* showed much lower values, as expected for organisms that primarily use organic osmotic solutes to provide osmotic balance. It must be noted that the apparent concentrations measured in both *S. ruber* and in *H. salinarum* were 3–4 times lower than those expected on account of the total salt concentration of the media employed. Leakage of ions during sample preparation may to some extent explain this discrepancy (see Discussion).

Analyses of intracellular organic solutes

Direct HPLC analysis of extracts of *S. ruber*, using a refractive index detector to identify organic solutes, did not show any organic osmotic compounds in significant concentrations. We succeeded in separating the glycine betaine from the large salt peak at 84% acetonitrile and at a relatively high dilution. In spite of the relatively low sensitivity thus obtained (detection limit 0.1 mM), we were able to estimate that the glycine betaine content increases from 0.2% to 0.35% and 0.44% of the dry weight in cells grown in 15%, 20%, and 25% salt, respectively. Based on these very low values, the possibility that glycine betaine might be involved in osmotic adaptation of the organism can be excluded. ^{13}C-NMR analyses of extracts of *S. ruber* strain M31, both when grown in the absence of

Table 2. Elemental analysis of *Salinibacter ruber* in the electron microscope and estimates of its intracellular ionic concentrations

	Salinibacter ruber	*Halobacterium salinarum*	*Halomonas elongata*	*Salinivibrio costicola*
Growth conditions	3.3 M NaCl, 250 g l^{-1} total salts	4.3 M NaCl, 262 g l^{-1} total salts	3.4 M NaCl, 210 g l^{-1} total salts, minimal medium	3.4 M NaCl, 206 g l^{-1} total salts, complex medium
Number of cells analyzed	25	20	27	39
Cell volume (μ^3)	1.85 ± 1.18	2.03 ± 0.84	0.39 ± 0.35	1.07 ± 0.48
Analyzed area (μ^2)	3.42 ± 1.73	3.09 ± 1.02	0.89 ± 0.37	1.96 ± 0.67
Elemental composition (fg $cell^{-1}$)				
C	170 ± 90	180 ± 80	81 ± 45	100 ± 110
N	30 ± 10	30 ± 10	16 ± 10	15 ± 16
O	100 ± 70	30 ± 10	12 ± 8	23 ± 26
P	5 ± 3	8 ± 3	2 ± 0.13	4 ± 4
S	8 ± 6	3 ± 1	0.7 ± 0.5	1.0 ± 0.7
Na	17 ± 13	10 ± 5	1.6 ± 1.4	2 ± 2
K	42 ± 27	70 ± 30	1.4 ± 1.0	3 ± 2
Mg	13 ± 9	2 ± 1	0.5 ± 0.3	9 ± 6
Cl	61 ± 44	80 ± 30	4.8 ± 3.4	12 ± 9
Apparent intracellular concentrations (M)				
Na	0.38 ± 0.11	0.22 ± 0.12	0.19 ± 0.09	0.06 ± 0.05
K	0.60 ± 0.13	0.93 ± 0.33	0.11 ± 0.05	0.07 ± 0.03
Mg	0.29 ±0.07	0.03 ± 0.02	0.06 ± 0.02	0.13 ± 0.15
Cl	0.91 ± 0.17	1.14 ± 0.31	0.39 ± 0.12	0.28 ± 0.14

For comparison, similar measurements were made with the halophilic Archaeon *Halobacterium salinarum* and the halophilic Bacteria *Halomonas elongata* and *Salinivibrio costicola*. Data presented are mean values ± standard deviation of the data collected by analyses of between 20 and 39 single cells

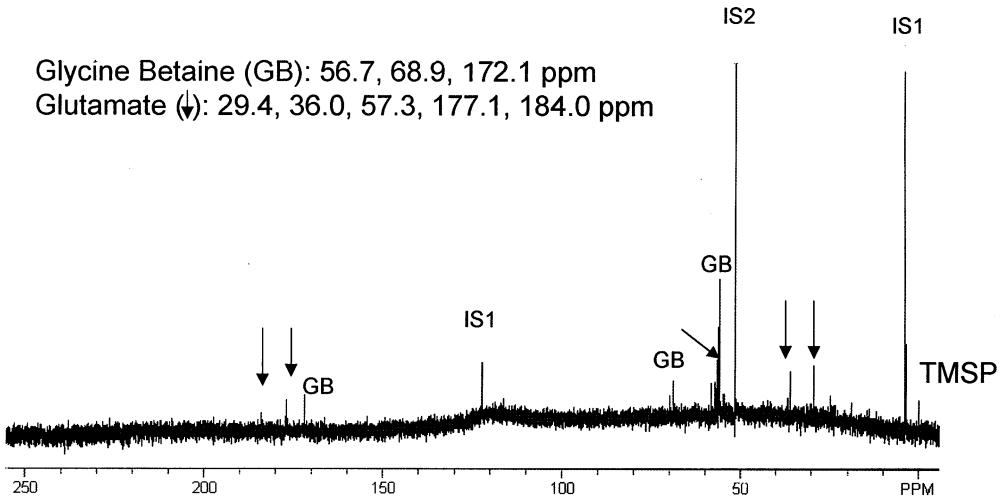

Fig. 1. ^{13}C-NMR pattern of the intracellular solutes of *Salinibacter ruber* 31. Signals could be attributed to the presence of glycine betaine (*GB*) and glutamate (*arrows*). *IS1* internal standard acetonitrile, *IS2* internal standard methanol (both at 30 µl ml^{-1}), *TMSP* trimethylsilylpropionate sodium salt (10 mg ml^{-1})

yeast extract and in 0.1% yeast-extract-containing media, only showed signs of glutamate and of glycine betaine at low, normal pool-size levels (Fig. 1). Additional weak and unidentified signals were detected in extracts of cells grown in the presence of yeast extract. In all cases the concentrations were far below those normally found in solute-producing organisms.

HPLC analysis of amino acids and other FMOC-reactive compounds showed that glutamate was the main intracellular amino acid, at the relatively low concentration of about 5.8 and 4.5 mg g dry $weight^{-1}$ in pellets of cells grown in 150 and in 250 g l^{-1} NaCl, respectively, corresponding to about 19 and 24 mg g cell $protein^{-1}$ (Fig. 2). When again assuming a value of 2.75 µl mg $protein^{-1}$ for these cells, these values would correspond with 47 and 59 mM intracellular glutamate. The only compound that increased in concentration with salinity was *N*-acetyllysine. However, its cytoplasmic levels were even lower than those of glutamate (from undetectable concentrations at 150 g l^{-1} NaCl to 1.2 mg g dry $weight^{-1}$ at 195 g l^{-1} NaCl or 6.5 mg g $protein^{-1}$ in cells grown in 250 g l^{-1} NaCl). Other, unknown, amino-reactive compounds were present, especially in cells grown in the presence of yeast extract, but their concentration was very low (below 2 mg per gram dry weight).

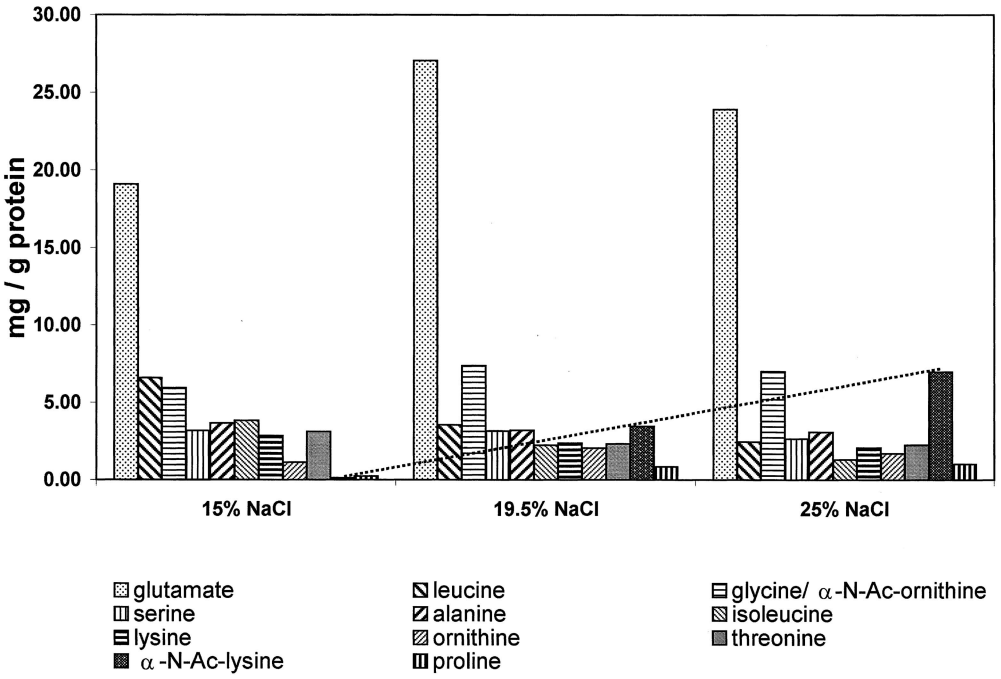

Fig. 2. Intracellular amino acid concentrations of *Salinibacter ruber* M31 growth in medium containing 15%, 19.5%, and 25% NaCl, as based on HPLC analysis of 9-fluorenylmethoxycarbonyl chloride (FMOC) derivatives. Per dry weight data were converted to per protein on the basis of the protein per dry weight assays

Discussion

Our conclusion that the Bacterium *Salinibacter ruber* uses KCl as osmotic solute was based both on our failure to detect organic osmotic solutes in significantly high concentrations and on the direct estimates of intracellular potassium ion concentrations. The ratio K^+/cellular protein has previously been used as an index to characterize halophilic Archaea (Pérez-Fillol and Rodríguez-Valera 1986). K^+/protein ratios ($g\,g^{-1}$) between 0.6 and 1.9 were reported in *Halobacterium salinarum* and between 0.5 and 1.3 in *Haloferax mediterranei*. These values would correspond to 15–49 µmol mg $protein^{-1}$ and to 13–33 µmol mg $protein^{-1}$, values somewhat higher than those found in the halophilic Archaea used in the present study.

X-ray microanalysis with the electron microscope has been used extensively in the elemental analyses of marine and other bacteria (Heldal et al. 1985; Norland et al. 1995). We have successfully employed the technique in the past to assess the intracellular ion concentrations in the anaerobic Bacterium *Halanaerobium praevalens* (Oren et al. 1997). In the present study we have ascertained the occurrence of intracellular ions in the molar range both in *S. ruber* and in the halophilic Archaeon *Halobacterium salinarum*. Concentrations of cations (predominantly K^+) and Cl^- were in the same order of magnitude in both. The fact that the apparent KCl concentration in *H. salinarum* cells was around 1 M rather than the 4–5 M expected (Christian and Waltho 1962; Matheson et al. 1976) may be due to certain artifacts inherent in the procedure, especially the possibility of the leakage of ions from the cells during centrifugation The apparently low intracellular K^+ and high Na^+ values calculated in the pellet extraction experiments may point to the same problem. The possibility that only part of the cell volume is osmotically active should also be taken into account.

To what extent the apparently high Na^+ concentrations within *S. ruber* cells (around 0.4 M, see Table 2) are valid or whether they may have been caused by artifacts inherent to the experimental procedures used cannot be ascertained at present. Each of the methods used in this study to estimate intracellular ionic concentrations has its potential problems. The fact that different values for the apparent intracellular ionic concentrations are obtained in the different experimental procedures used illustrates this fact, which is also well known from other studies (see e.g., Ventosa et al. 1998).

Salinibacter shares with the halophilic Archaea of the order Halobacteriales a large excess of acidic amino acids (Oren and Mana 2002). Acidic residues are more highly hydrated than other amino acids and can coordinate the organization of a hydrated salt ion network at the surface of the protein (Dennis and Shimmin 1997; Dym et al. 1995; Eisenberg et al. 1992; Lanyi 1974; Madigan and Oren 1999). *S. ruber* also shares with the halophilic Archaea a low content of hydrophobic amino acids and a high content of serine (Oren and Mana 2002), properties considered to be a characteristic feature of halophilic proteins (Dennis and Shimmin 1997; Lanyi 1974). Accordingly, the intracellular enzymes of *S. ruber* are able to function in the presence of high salt concentrations (Oren and Mana 2002). It thus appears that a similar strategy has been developed in these two types of organisms that share a common environment: the NaCl-saturated crystallizer ponds of solar salterns for the commercial production of salt from seawater. *S. ruber* shows additional similarities with the Halobacteriales, such as its extremely high salt requirement (no growth being observed below 15% NaCl) (Antón et al. 2002) and a red pigmentation (Oren and Rodríguez-Valera 2001).

The absence of significant quantities of organic osmotic solutes in *S. rubrum* was unexpected in view of its phylogenetic position, being related to the *Cytophaga–Flavobacterium* branch of the domain Bacteria. All the halophilic and halotolerant aerobic Bacteria characterized so far primarily use organic compatible solutes, and generally contain relatively low concentrations of inorganic ions (Galinski 1995; Ventosa et al. 1998). Moreover, the closest known relative of *S. ruber*, based on 16S rRNA sequence comparison, is the marine thermophile *Rhodothermus marinus* isolated from submarine hot springs. This organism uses unusual organic solutes – α-mannosylglycerate and α-mannosylglyceramide – to provide osmotic balance (Silva et al. 1999). The use of such organic solutes to provide osmotic balance is probably not feasible for an organism such as *S. ruber*, which grows optimally at NaCl concentrations of around 4 M because of solubility limitations. With the exception of glycerol, which is never used in the prokaryote world as a compatible solute, none of the known organic osmotic solutes is soluble at the concentrations needed to balance 8 M ions. The use of cocktails of organic solutes may be another option. *Halorhodospira* species provide a good example, having glycine betaine, ectoine, and trehalose (Galinski and Herzog 1990). Glycine betaine is a relatively favorable organic solute, as it is small and energetically relatively inexpensive to produce. However, only a few halophilic microorganisms are able to synthesize the compound. We did not detect any of the compounds mentioned in significant concentrations in *S. ruber*. The possibility that *S. ruber* may contain unknown organic osmolytes can be excluded because our ^{13}C spectra did not show any strong signals.

There is only one additional group of Bacteria known that uses KCl rather than organic solutes to provide osmotic balance: the anaerobic fermentative members of the order Halanaerobiales (low G+C branch of the Firmicutes), families Halanaerobiaceae and Halobacteroidaceae (Oren 1986; Oren et al. 1997; Rengpipat et al. 1988). In this case it was argued that, due to the low amounts of energy generated during fermentation processes, the use of the energetically favorable KCl rather than expensive-to-produce organic solutes is a bioenergetic necessity (Oren 1999). No such bioenergetic constraints should be present in the case of the obligatory aerobic *S. ruber*.

There is a surprising similarity between the physiology of the extremely halophilic Bacteria of the genus *Salinibacter* and the Archaea of the family Halobacteriaceae. Both groups are aerobic heterotrophs, both maintain high intracellular K^+ concentrations, and both possess enzymes that are functional at high salt concentrations. A comparative genomic analysis of *Salinibacter* and members of the Halobacteriaceae may therefore prove to be of great interest, and is expected to shed new light on the adaptations of halophilic microorganisms at NaCl concentrations approaching saturation.

Acknowledgments Support for this study was obtained from the Israel Science Foundation, founded by the Israel Academy of Sciences and Humanities, and the "Improving Human Potential Program" of the European Union through contract no. HPRI-1999-CT-00056 (to A.O.). Special thanks are due to Mr. Egil Ericksen for his assistance with the operation of the electron microscope and elemental analysis system at the University of Bergen.

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