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ORIGINAL PAPER

The amino acid composition of proteins from anaerobic halophilic bacteria of the order Halanaerobiales

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Abstract We performed a comparative analysis of the genome sequences of three anaerobic halophilic fermentative bacteria belonging to the order Halanaerobiales: *Halanaerobium praevalens*, the alkaliphilic "Halanaerobium hydrogeniformans", and the thermophilic *Halothermothrix orenii* to assess the amino acid composition of their proteins. Members of the Halanaerobiales were earlier shown to accumulate KCl rather than organic compatible solutes for osmotic balance, and therefore the presence of a dominantly acidic proteome was predicted. Past reports indeed showed a large excess of acidic over basic amino acids in whole-cell hydrolysates of selected members of the order. However, the genomic analysis did not show unusually high contents of acidic amino acids or low contents of basic amino acids. The apparent excess of acidic amino acids in these anaerobic halophiles reported earlier is due to the high content in their proteins of glutamine and asparagine, which yield glutamate and aspartate upon acid hydrolysis. It is thus suggested that the proteins of the Halanaerobiales, which are active in the presence of high intracellular KCl concentrations, do not possess the typical acidic signature of the 'halophilic' proteins of the Archaea of the order Halobacteriales or of the extremely halophilic bacterium *Salinibacter*.

Keywords Halanaerobiaceae · Acidic proteins · Halophilic · Anaerobic · Osmotic adaptation

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Abbreviations

Han.	Halanaerobium
Hbs.	Halobacteroides
Hbt.	Halobacterium
Hmn.	Halomonas
Htx.	Halothermothrix

Introduction

Two fundamentally different strategies have been identified that enable prokaryotic microorganisms to live at high-salt concentrations. The most commonly found mode of osmotic adaptation is the biosynthesis of organic osmotic solutes ('compatible solutes'), such as glycine betaine, ectoine, and hydroxyectoine, or their accumulation from the medium. Intracellular salt concentrations are maintained at a level far below that of the outside medium, and no far-reaching adaptations of the intracellular machinery are needed to enable the cells to function in high-osmolarity environments. The intracellular concentrations of the solutes can be rapidly adjusted, so that microorganisms using this strategy can often adapt to life at a wide range of salinities (Oren 2002, 2011).

The second strategy involves the accumulation of ions, mainly K^+ and Cl^-, to provide osmotic balance. This 'salt-in' strategy requires special adaptations of the intracellular proteome as all enzymes must function in the presence of molar concentrations of KCl. In general, such halophilic proteins require salt for structural stability and activity, so that microorganisms that use this mode of osmotic adaptation require high-salt concentrations and have limited abilities to cope in low-salt media. The 'salt-in' strategy

was thus far found in a few groups of prokaryotes only: the aerobic extremely halophilic Archaea of the family Halobacteriaceae (Oren 2002, 2006a), the red aerobic *Salinibacter* (Bacteroidetes) (Antón et al. 2002; Oren et al. 2004), and a phylogenetically coherent group of obligatory anaerobic bacteria of the order Halanaerobiales, affiliated with the low G + C branch of the Firmicutes (Oren 2006b).

The order Halanaerobiales currently contains two families: the Halanaerobiaceae and the Halobacteroidaceae (Rainey et al. 1995). Representatives were isolated from a wide variety of anaerobic hypersaline environments including sediments of Great Salt Lake, Utah, Salton Sea, California, the Dead Sea, saltern ponds, oil wells and petroleum reservoir fluids, hypersaline lakes and lagoons in the Crimea and Senegal, and alkaline hypersaline lakes such as Lake Magadi, Kenya and Big Soda Lake, Nevada. At the time of writing (February 2012) the order contained 13 genera with 27 species with names that have standing in the nomenclature of prokaryotes. All are strict anaerobes. Most thrive optimally at NaCl concentrations around 10–15 %, and some even grow in media saturated with NaCl. Most species grow fermentatively on sugars, producing acetate, ethanol, hydrogen, CO_2, and other fermentation products such as butyrate, lactate, or propionate. Some genera (*Acetohalobium*, *Natroniella*) have a homoacetogenic metabolism, producing acetate as the main end product of their energy metabolism. Others (*Selenihalanaerobacter shriftii*) grow by anaerobic respiration with selenate or nitrate as electron acceptor. Most are neutrophilic, some species are alkaliphiles, and one (*Halothermothrix orenii*) is a thermophile that grows optimally at 60 °C and up to 68 °C at salt concentrations as high as 20 %. It was isolated from a warm saline lake in Tunisia (Cayol et al. 1994). The biology of the group was reviewed by Oren (2006b) and by Kivistö and Karp (2011).

Accumulation of organic osmotic solutes could not be demonstrated in the cytoplasm of most members of the Halanaerobiales tested (Oren 1986; Rengpipat et al. 1988), but high intracellular concentrations of Na^+, K^+ and Cl^- were measured in *Halanaerobium praevalens* (Oren 1986; Oren et al. 1997), *Halobacteroides halobius* (Oren 1986), *Han. acetethylicum* (Rengpipat et al. 1988), and *Natroniella acetigena* (Detkova and Pusheva 2006), high enough to be approximately isotonic with the medium. Intracellular enzymes of these organisms function well in the presence of high-salt concentrations (see "Results and discussion"). It was thus concluded that the Halanaerobiales use the 'salt-in' strategy of osmotic adaptation (Oren 2006b; Kivistö and Karp 2011).

It is known since the 1970s that the proteins of the halophilic Archaea of the family Halobacteriaceae have a large excess of acidic amino acids (glutamate, aspartate) over basic amino acids (lysine, arginine). Such halophilic proteins have a strong negative charge at the physiological pH (Reistad 1970; Lanyi 1974; Mevarech et al. 2000). The excess of acidic versus basic amino acids can therefore be used to assess the extent to which proteins may be adapted to function in a high-salt environment. *Salinibacter*, a KCl accumulating member of the Bacteria (Oren et al. 2004) also has an acidic proteome (Oren and Mana 2002), a finding that was confirmed by the analysis of its genome (Mongodin et al. 2005).

A few attempts have been made in the past to assess the acidic nature of the proteins of the Halanaerobiales by acid hydrolysis of the cells and chromatographic quantification of the amino acids released (Table 1). These studies encompassed *Han. praevalens*, *N. acetigena* (Halanaerobiaceae) and *Hbs. halobius*, *Han. saccharolyticum*, and *Sporohalobacter lortetii* (Halobacteroidaceae) (Oren 1986; Detkova and Boltyanskaya 2006). The overall conclusion was that the bulk protein of these anaerobic halophiles has a strongly acidic nature, comparable to that of the proteins of *Halobacterium* and related extremely halophilic Archaea. However, it must be taken into account that the procedure included acid hydrolysis, converting asparagine to aspartate and glutamine to glutamate.

As the analysis of the genome of *Htx. orenii* did not show a pronounced acidic nature of the encoded proteins (Mijts and Patel 2001; Mavromatis et al. 2009), we performed a comparative analysis of three published genomes of members of the Halanaerobiales: *Han. praevalens*, "Halanaerobium hydrogeniformans", and *Htx. orenii*.

Methods

The following genomes of members of the Halanaerobiales were used in our analyses: *Han. praevalens* GSL^T (Ivanova et al. 2011), being the type strain of the type species of the type genus of the order, the thermophilic *Htx. orenii* H168^T (Mavromatis et al. 2009), and the genome of a haloalkaliphilic hydrogen-producing organism known as "Halanaerobium hydrogeniformans", earlier designated as "Halanaerobium sapolanicus" (Brown et al. 2011). As far as we could ascertain, this organism cannot currently be obtained from culture collections and except for the genome sequence little information is available except that it was isolated from the alkaline hypersaline and sulfide-rich Soap Lake, Washington, USA, that it grows optimally at pH 11, 7 % NaCl and 33 °C, and that it produces acetate, formate and hydrogen. For comparison we included the genome sequences of *Halobacterium* NRC1 (Euryarchaeota, extreme halophile that accumulates KCl) (Ng et al. 2000), *Salinibacter ruber* M31^T (Bacteroidetes, using a similar mode of osmotic adaptation as *Halobacterium*) (Mongodin et al. 2005), *Halomonas elongata* 1H9^T (Gammaproteobacteria, producing ectoine as compatible solute (Schwibbert et al. 2011), and the non-halophiles

Table 1 Acidic and basic amino acids in members of the Halanaerobiales (printed in boldface) compared to other extremely halophilic, moderately halophilic and non-halophilic prokaryotes, as reported in the literature and based on the analysis of amino acids in acid hydrolysates of whole cells

Name	Affiliation and properties	$(D + N + E + Q)$/(total amino acids) × 100 %	$(D + N + E + Q - K - R)$/(total amino acids) × 100 %	Reference
***Halanaerobium praevalens* GSL^T**	**Halanaerobiaceae**	**27.7**	**16.0**	Oren (1986)
***Halobacteroides halobius* MD-1^T**	**Halobacteroidaceae**	**25.8**	**12.2**	
***Sporohalobacter lortetii* MD-2^T**	**Halobacteroidaceae**	**26.8**	**14.7**	
Halobacterium salinarum R1[1]	Extremely halophilic archaeon	27.9	13.2	
Escherichia coli B	Non-halophilic	20.0	6.0	
***Halanaerobium saccharolyticum* Z-7787^T**	**Halanaerobiaceae**	**25.7**	**18.9**	Detkova and Boltyanskaya (2006)
***Natroniella acetigena* Z-7937^T**	**Halobacteroidaceae**	**30.9**	**24.0**	
Acetobacterium paludosum Z-4092^T	Non-halophilic	20.8	12.4	
Halomonas campisalis Z-7398-2	Moderate halophile that uses organic osmotic solutes	23.2	15.7	
Halobacterium salinarum strain 1	Extremely halophilic archaeon	26.8	19.1	Reistad (1970)
Pseudomonas fluorescens	Non-halophilic	20.9	9.1	
Haloferax mediterranei R4^T	Extremely halophilic archaeon	26.0	19.3	Ghandbhir et al. (1995)
Halomonas elongata 1H9^T	Moderate halophile that uses organic osmotic solutes	22.3	12.5	
Escherichia coli B/r	Non-halophilic	20.0	7.8	

Asparagine (*N*) and glutamine (*Q*) are converted to asparatate (*E*) and glutamate (*D*), respectively, during acid hydrolysis, and these are therefore included in the percentages of acidic amino acids

^{T} = type strain, K = lysine, R = arginine

Chlorobaculum tepidum TLS^T and *Bacteroides fragilis* NCTC 9343^T. The accession numbers of the sequences are given in Table 2.

From the genome annotations we extracted those sequences encoding proteins or putative proteins, and calculated for each the percentages of acidic amino acids (Glu, Asp), basic amino acids (Lys, Arg), and the excess of acidic over basic amino acids. For each protein sequence we also predicted the p*I* value (isoelectric point), using the programs in the Galaxy platform (http://main.g2.bx.psu.edu) (Blankenberg et al. 2010; Giardine et al. 2005; Goecks et al. 2010).

Results and discussion

In view of the above-discussed assays that showed a large excess of acidic over basic amino acids in whole-cell hydrolysates of selected members of the order Halanaerobiales (Table 1), we expected to find a similar bias toward acidic proteins in the proteome encoded by the genomes analyzed. However, we did not find such preferential use of acidic amino acids and no particularly low content of basic amino acids (Table 2). *Han. praevalens* and *Htx. orenii* both showed a low excess of acidic amino acids: 0.4 mol%, a value similar to the non-halophilic bacteria included in the comparison, and very low compared to 7.5 mol% in *Halobacterium*, and 4.1 mol% in *Salinibacter*, both halophiles that accumulate KCl for osmotic balance. Earlier sequence analysis of the ribosomal A-protein of *Han. praevalens* did not show a particularly high content of acidic amino acids (Matheson et al. 1987). The lack of a pronounced acidic nature of the proteins of *Htx. orenii*, earlier attributed to the high temperature optimum of this organism (Mijts and Patel 2001; Mavromatis et al. 2009), thus appears to be a general character of the group, not

Table 2 Analysis of the amino acids of the proteins encoded by the genomes of three members of the Halanaerobiales (*Halanaerobium praevalens*, "Halanaerobium hydrogeniformans", and *Halothermothrix orenii*), as compared to the proteins encoded by the genomes of the extremely halophilic archaeon *Halobacterium* NRC-1, the extremely halophilic bacterium *Salinibacter ruber* (Bacteroidetes), the moderately halophilic *Halomonas elongata* (Gammaproteobacteria), and the non-halophilic *Chlorobaculum tepidum* and *Bacteroides fragilis*

Name	GenBank/genome on line database accession number	Total number of proteins	Total amino acids	*D*	*E*	*Q*	*N*	*K*	*R*	(*D* + *E*) (mol%)	(*Q* + *N*) (mol%)	(*K* + *R*) (mol%)	(*D* + *E*) − (*K* + *R*) (mol%)	(*D* + *Q*+*E* + *N*) − (*K* + *R*) (mol%)
Halanaerobium praevalens GSL^T	CP002175 Gc01415	2,110	687,192	35,916	53,329	24,515	39,130	62,921	23,651	13.0	9.3	12.6	0.4	9.6
"Halanaerobium hydrogeniformans"	CP002304	2,391	780,822	46,305	65,350	23,142	42,682	65,029	30,838	14.3	8.4	12.3	2.0	10.4
Halothermothrix orenii H168^T	CP001098 Gc01049	2,365	749,048	41,213	55,903	19,950	38,897	59,023	35,440	13.0	7.9	12.6	0.4	8.2
Halobacterium NRC1	NC_00260	2,675	750,764	66,763	52,501	16,713	20,833	13,417	49,219	15.9	5.0	8.3	7.5	12.5
Salinibacter ruber M31^T	CP000159	2,845	1,015,900	71,009	71,422	36,029	24,902	21,596	78,778	14.0	6.0	9.9	4.1	10.1
Halomonas elongata 1H9^T	NC_014532	3,474	1,171,599	71,153	78,309	42,383	28,176	27,808	89,358	12.8	6.0	10.0	2.8	8.8
Chlorobaculum tepidum TLS^T	NR_044685	2,252	630,535	32,599	42,232	19,757	21,558	33,208	38,222	11.9	6.6	11.3	0.5	7.1
Bacteroides fragilis NCTC 9343^T	NC_003228	4,299	1,549,617	83,657	100,836	53,419	78,187	102,703	73,410	11.9	8.5	11.4	0.6	9.0

Listed are the numbers of acidic amino acids glutamate (*D*) and aspartate (*E*), the basic amino acids lysine (*K*) and arginine (*R*), as well as the amide amino acids glutamine (*Q*) and asparagine (*N*), relevant for the calculations discussed in the text. The last five columns present calculations of the percentages of acidic (whether or not including the amides) and basic amino acids and the excess of acidic amino acids over basic amino acids

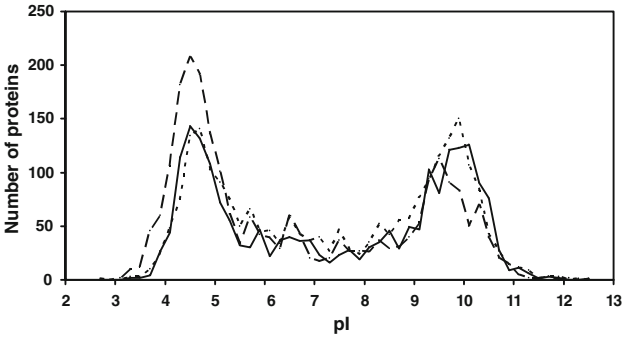

Fig. 1 Distribution of p*I* values at 0.2 intervals for predicted proteins encoded by the genomes of *Halanaerobium praevalens* (2,110 proteins; *solid line*), "Halanaerobium hydrogeniformans" (2,391 proteins, *dashed line*), and *Halothermothrix orenii* (2,365 proteins, *dotted line*)

connected with the need for the enzymes to function at high temperatures. The alkaliphilic "Halanaerobium hydrogeniformans" had a slightly higher excess of acidic amino acid residues: 2.0 mol%, which is still lower than the value of 2.8 mol% for the moderate halophile *Hmn. elongata*, an organism that synthesizes or accumulates organic osmotic solutes. The slightly elevated average acidity of the *Halomonas* proteins may at least in part be due to the need for its periplasmic proteins to function also at high-salt concentrations. The predicted p*I* values of selected proteins encoded by the genome of the phylogenetically related *Chromohalobacter salexigens* showed most of its proteins to be no more acidic than comparable proteins from non-halophiles, with the exception of periplasmic proteins that are exposed to the high salinity of the medium (Oren et al. 2005).

The distribution of the predicted p*I* values of the proteins encoded by the genomes of the three members of the Halanaerobiales (Fig. 1) was also very similar to the data presented by Mongodin et al. (2005) for the non-halophiles *C. tepidum* and *B. fragilis*: a bimodal distribution with peaks around 4.6–4.8 and 9.8–10.2. In "Han. hydrogeniformans" the relative size of the low-p*I* proteins peak is somewhat increased, as expected from its slightly higher percentage of acidic amino acids. The location of the two pI peaks in the distribution of the proteins is shifted to somewhat lower values for the Halanaerobiales. This p*I* distribution should be compared to the presence of dominant peaks around p*I* 4.5 and 4.8 for *Halobacterium* and *Salinibacter*, respectively (Mongodin et al. 2005). Thus, in spite of their halophilic properties, the Halanaerobiales do not follow the general trend of increased average protein acidity with increased salinity of the environment (Rhodes et al. 2010), a trend that was already noted in a microbial mat growing at the relatively low-salt concentration of 9 % in Guerrero Negro, Mexico (Kunin et al. 2008). Most microorganisms thriving at that salinity

are expected to use organic osmotic solutes and do therefore not require far-going adaptations of the intracellular proteome.

Our genomic analysis thus did not confirm the earlier reported apparent excess of acidic amino acids in the Halanaerobiales based on the analysis of the bulk cellular protein. Although a close correlation can be expected between the data obtained using the two approaches, it is impossible to directly compare the results: the analysis presented in Table 2 is based on a single copy of every protein encoded, while the bulk protein will contain some proteins in many copies, others in a few copies only, while still other proteins encoded by the genome may not be expressed at all under the growth conditions used. But even if the more acidic proteins are preferentially synthesized, the discrepancies between the data are still too large. A far more plausible explanation for the apparent high excess of acidic amino acids in cellular bulk protein, as documented in Table 1, can be found in the relatively high content of the amide amino acids glutamine and asparagine (Table 2), which lose their amide group upon acid hydrolysis during sample preparation for amino acid analysis, yielding glutamate and aspartate.

Inspection of the annotated genomes of the three members of the Halanaerobiales did not yield evidence for the possible biosynthesis of organic osmotic solutes such as glycine betaine or ectoine by these bacteria. In the genome of *Htx. orenii* a gene encoding sucrose phosphate synthase was identified, which points to the possibility that sucrose may be formed. It remains to be assessed whether the gene is expressed during salt stress and whether sucrose may accumulate in the cytoplasm as an osmotic solute. Genes encoding for glycine betaine/L-proline transport were found in all three genomes, but such genes are present in nearly all bacterial genomes. Glycine betaine was indeed identified in cells of *Orenia salinaria* (Halobacteroidaceae) when grown in media containing yeast extract (Mouné et al. 2000). But altogether there is no evidence to support that organic osmolytes rather than inorganic ions provide osmotic balance in the Halanaerobiales. All cytoplasmatic enzymes tested function well in the presence of molar concentrations of salts, and many have lowered activities when tested in the absence of salt. Examples are the fatty acid synthetase complex of *Han. praevalens* (Oren and Gurevich 1993), glyceraldehyde-3-phosphate dehydrogenase, NAD-linked alcohol dehydrogenase, pyruvate dehydrogenase, and methyl viologen-linked hydrogenase from *Han. acetethylicum* (Rengpipat et al. 1988), carbon monoxide dehydrogenase of *N. acetigena* (Detkova and Boltyanskaya 2006; Detkova and Pusheva 2006), and the hydrogenase and carbon monoxide dehydrogenase of *Acetohalobium arabaticum* (Zavarzin et al. 1994).

In summary, the proteins of the Halanaerobiales, which are active in the presence of high intracellular KCl concentrations, do not possess the typical acidic signature of the 'halophilic' proteins of the Archaea of the order Halobacteriales or of the extremely halophilic bacterium *Salinibacter*. An in-depth study of their special properties is thus recommended to increase our understanding of the different strategies used in the prokaryote world to enable growth at high-salt concentrations.

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