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Aharon Oren · Frank Larimer · Paul Richardson
Alla Lapidus · Laszlo N. Csonka

How to be moderately halophilic with broad salt tolerance: clues from the genome of *Chromohalobacter salexigens*

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Abstract We analyzed the amino acid composition of different categories of proteins of the moderately halophilic bacterium *Chromohalobacter salexigens*, as deduced from its genome sequence. Comparison with non-halophilic representatives of the γ-Proteobacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*) shows only a slight excess of acidic residues in the cytoplasmic proteins, and no significant differences were found in the acidity of membrane-bound proteins. In contrast, a very pronounced difference in mean pI value was observed for the periplasmic binding proteins of the ABC transport systems of *C. salexigens* and the non-halophiles *E. coli* and *P. aeruginosa*. *V. cholerae*, which is adapted to life in brackish water, showed intermediate values. The findings suggest that there is a major difference between the proteins of the moderate halophile *C. salexigens* and non-halophilic bacteria in their periplasmic proteins, exemplified by the substrate binding proteins of transport systems. The highly acidic nature of these proteins may enable them to function at high salt concentrations. The evolution of highly salt-tolerant prokaryotes may have depended on an increase in acidity of the proteins located external to the cytoplasmic membrane, enabling effective transport of nutrients into the cell.

Keywords *Chromohalobacter salexigens* · Halophilic · Genome sequence · Periplasmic binding proteins · Isoelectric point

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A. Oren (✉)
The Institute of Life Sciences, and the Moshe Shilo Minerva Center for Marine Biogeochemistry,
The Hebrew University of Jerusalem,
91904 Jerusalem, Israel
E-mail: orena@shum.cc.huji.ac.il
Tel.: +972-2-6584951
Fax: +972-2-6528008

F. Larimer
Genome Analysis and Systems Modeling,
Life Sciences Division,
Oak Ridge National Laboratory,
Oak Ridge, TN 37831,USA

P. Richardson · A. Lapidus
DOE Joint Genome Institute,
Walnut Creek, CA 94598, USA

L. N. Csonka
Department of Biological Sciences,
Purdue University, West Lafayette,
IN 47907-1392, USA

Introduction

There are two strategies that enable microorganisms to grow at high salt concentrations. Some groups balance the high osmolality of their environment with high intracellular concentrations of KCl. Examples are the aerobic Archaea of the family Halobacteriaceae, the aerobic bacteria of the genus *Salinibacter*, and the anaerobic fermentative bacteria of the order Halanaerobiales (Lanyi 1974; Oren 1986, 2002; Oren et al. 2002). Adaptation of all intracellular proteins is necessary for such organisms to function, and this is reflected in a large excess of acidic over basic residues and a low content of hydrophobic amino acids (Lanyi 1974; Oren 1986; Madern et al. 2000; Mevarech et al. 2000). Since Reistad (1970) reported the presence of a large excess of acidic amino acid residues in the bulk protein of *Halobacterium* and *Halococcus* more than 30 years ago, much information has accumulated on the special properties of enzymes and other proteins that have to be stable and active in the presence of high salt concentrations. The review by Lanyi (1974) shows the general trend: the typical signature of a halophilic protein consists of an unusually large content of acidic amino acids, a low content of basic amino acids, and a relatively low proportion of hydrophobic amino acids, which is offset by a higher than usual content of the 'borderline hydrophobic' amino acids Ser and Thr. The genome of *Halobacterium* NRC-1, a representative par

excellence of the extremely halophilic *Archaea* that balance the outside salt concentration by accumulating molar concentrations of KCl intracellularly, confirms the earlier analyses (Ng et al. 2000). Different models have been devised to explain how such an amino acid composition may lead to the halophilic behavior of proteins (Lanyi 1974; Britton et al. 1998; Elcock and McCammon 1998; Madern et al. 2000; Mevarech et al. 2000). Such models are valuable, even when in-depth analysis of the properties of selected salt-dependent proteins suggests that a variety of mechanisms may be operative on an individual basis. Evolutionary models can explain the transition from non-halophilic to increasingly halophilic proteins (Dennis and Shimmin 1997).

Many other halophilic and halotolerant microorganisms keep their intracellular ionic concentration at a low level (for definitions of the terms halophilic and halotolerant, and the impossibility to strictly define such terms in view of the continuum of salt requirement and salt tolerance ranges in the microbial world, see Kushner 1978). Instead they use organic solutes to provide the necessary osmotic balance. These organic 'compatible' solutes, such as glycine betaine, ectoine, simple sugars, and many others (Galinski 1995; Oren 2002), are much less disruptive to normal functioning of enzymes than high concentrations of KCl, and accordingly less far-reaching adaptations are required in the protein structure. Many of these halophiles (e.g. the *Halomonas—Chromohalobacter* group in the γ-Proteobacteria branch of the bacteria) can grow over a remarkably broad range of salt concentrations, while adjusting the intracellular organic solute concentration (Ventosa et al. 1998). The bulk amino acid composition of such organisms does not show the typical halophilic bias of the *Halobacterium* proteins, although a slight excess of acidic amino acids has been observed in different representatives of the group as compared to non-halophilic bacteria (Gandbhir et al. 1995; Oren 1995).

A nearly complete genome sequence of the moderately halophilic bacterium *Chromohalobacter salexigens* DSM 3043 has recently been obtained. *C. salexigens* is a metabolically versatile, moderately halophilic bacterium, isolated from a solar saltern on Bonaire, Netherlands Antilles. It is an aerobic chemoorganotroph that grows on a wide range of simple carbon compounds at NaCl concentrations between 0.5 M and 4 M, with an optimum at 2–2.5 M (Ventosa et al. 1989, 1998; Cánovas et al. 1996; Arahal et al. 2001). The availability of its sequence enables a search for halophilic signatures in different categories of proteins encoded by its genome. We analyzed the amino acid composition of different categories of *C. salexigens* proteins, as deduced from this genome sequence, comparing their amino acid composition with equivalent proteins of related non-halophilic bacteria. The results, as presented below, provide valuable clues to the functioning of moderately halophilic bacteria with a distinct salt requirement.

Materials and methods

Genome sequences of *Chromohalobacter salexigens* and of other halophilic and non-halophilic prokaryotes

A nearly complete genome sequence of *C. salexigens* DSM 3043 has recently been determined by the Joint Genome Institute of the US Department of Energy, using standard genome sequencing techniques. A draft sequence of 47 major contigs and their annotation is now publicly available (http://genome.jgi-psf.org/microbial/index.html). The *E. coli* K12 genome sequence was derived from http://www.tigr.org/ and its annotation from http://www.genome.wisc.edu/pub/sequence/U00096.2.gbk/. Genome data for *P. aeruginosa*, *V. cholerae*, and *Halobacterium* NRC-1 were taken from http://www.pseudomonas.com/current_annotation.asp/, http://www.tigr.org/, and http://zdna2.umbi.umd.edu/cgi-bin/haloweb/nrc1.pl?operation=nrc1/, respectively.

Calculation of pI values and amino acid composition of putative proteins

Lists of putative proteins, as deduced from the annotated genome sequences, used in the analyses summarized in Tables 1, 2 and 3, can be obtained from the corresponding author upon request.

Predicted pI values of the proteins were calculated by the algorithm given in http://www.emb l-heidelberg.de/cgi/pi-wrapper.pl/. The N-terminal Met residue was not taken into account for amino acid composition calculations.

Results and discussion

The isolectric point (pI) provides a simple indication of the acidic nature of proteins. We compared the calculated pI values of selected proteins of *C. salexigens* with orthologs and other related proteins from three γ-Proteobacteria, *E. coli* and *P. aeruginosa* (non-halophiles), and *V. cholerae* (which is adapted to life in brackish water), and from an extremely halophilic archaeon, *Halobacterium* NRC-1 (Table 1). The average pI of three categories of cytoplasmic proteins (enzymes of central metabolic pathways, structural ribosomal proteins, and the membrane-associated ATPase components of ABC transport systems) is consistently slightly lower in *C. salexigens* than in *E. coli* and *P. aeruginosa*. The values for *V. cholerae* are intermediate between the moderate halophile and the two non-halophiles. In all cases, the pI values are much higher than those of the orthologs in the extremely halophilic *Halobacterium*, as expected from the overall much higher acidic amino acid content of the bulk protein of the latter. A slightly increased acidity of the ribosomal proteins of *C. salexigens* is in agreement with earlier reports on the ribosomes of the closely

Table 1 Mean pI values of different categories of proteins of *Chromohalobacter salexigens*, as compared with their counterparts form the non-halophilic *Escherichia coli* K-12, *Pseudomonas aeruginosa* PA01, *Vibrio cholerae* O1 El Tor N16961, and the extremely halophilic archaeon *Halobacterium* sp. NRC-1

Gene category	*C. salexigens*	*E. coli*	*P. aeruginosa*	*V. cholerae*	*Halobacterium*
Central metabolism	5.10 ± 0.34 (12)	5.66 ± 0.49 (12)	5.80 ± 0.55 (12)	5.54 ± 0.44 (12)	4.22 ± 0.14 (6)
Ribosomes	10.10 ± 2.15 (53)	10.41 ± 1.67 (53)	10.37 ± 1.81 (53)	10.28 ± 1.88 (53)	5.81 ± 2.56 (55)
ATP binding components of ABC transport systems	6.67 ± 1.47 (50)	6.93 ± 1.63 (73)	7.06 ± 1.62 (62)	6.90 ± 1.41 (54)	4.42 ± 1.10 (26)
Permease components of ABC transport systems	9.19 ± 1.56 (68)	9.18 ± 1.44 (77)	9.24 ± 1.49 (60)	8.65 ± 1.75 (53)	6.68 ± 2.46 (27)
Periplasmic binding components of ABC transport systems	4.54 ± 1.13 (55)	6.81 ± 1.56 (59)	7.28 ± 1.33 (44)	5.68 ± 1.03 (39)	4.11 ± 0.14 (6)

Analysis of enzymes of the central metabolism was limited to orthologs identified in the genomes of all four γ-Proteobacteria. All structural ribosomal proteins were included in the analysis, as were all periplasmic, membrane-bound, and cytoplasmic components of the ABC transporters annotated in the genomes. The table gives the mean values and standard deviation, and the number of proteins on which the calculation was based are given in brackets

related *Chromohalobacter canadensis* and other moderate halophiles (Falkenberg et al. 1976, 1986; Matheson et al. 1978). The membrane-bound permease components of the ABC transporters are no more acidic in *C. salexigens* than in any of the non-halophiles. As these proteins are embedded within the cytoplasmic membrane, their structure is expected to be influenced more by the properties of the membrane than by the ionic environment inside or outside the cells. It may also be noted that the *Halobacterium* counterparts of these proteins are on the average far less acidic than any of the other protein categories from this organism.

In contrast, a very pronounced difference in mean pI value was observed for the periplasmic binding proteins of the ABC transport systems of *C. salexigens* and the two non-halophiles: about 2.5 pH units below the values for *E. coli* and *P. aeruginosa*. The large number of genes encoding such proteins in the four bacterial genomes included in the comparison makes the result highly significant. This category of proteins is almost as acidic in *C. salexigens* as in *Halobacterium* (in which only very few such proteins have been annotated and which does not possess an outer membrane). Again, *V. cholerae* showed values intermediate between those of *C. salexigens* and the non-halophiles. As the outer membrane of the Gram-negative bacteria does not present a barrier for ions, the periplasmic binding proteins are exposed to the full salinity of the medium. The G + C composition of the genomes of *C. salexigens* and *P. aeruginosa* are 64 and 66%, respectively, and therefore, the high G + C

Table 2 Contents of acidic and basic amino acids (in mole-percent) in different protein fractions of *C. salexigens* as compared to *E. coli*, *P. aeruginosa*, *V. cholerae*, and *Halobacterium* NRC-1

	Glu	Asp	Lys	Arg
Ribosomes				
C. salexigens (53; 7196)	6.6	4.7	**8.7**	8.9
E. coli (53; 7273)	6.4	4.5	9.4	8.7
P. aeruginosa (53; 7025)	6.4	4.5	9.4	8.7
V. cholerae (53; 7016)	6.7	4.1	9.5	8.4
Halobacterium (55; 8061)	**9.9**	**9.1**	**4.4**	**7.7**
Substrate binding proteins				
C. salexigens (55; 19036)	**6.9**	**7.9**	**2.8**	5.0
E. coli (59; 21232)	5.2	6.0	6.9	3.7
P. aeruginosa (44; 15899)	5.5	6.1	6.1	5.4
V. cholerae (39; 14055)	5.8	5.6	6.2	3.5
Enzymes of the central metabolism				
C. salexigens (12; 7187)	7.0	**6.9**	**4.1**	6.1
E. coli (12; 7102)	6.6	6.1	5.1	5.6
P. aeruginosa (12; 7138)	6.9	5.8	4.8	6.1
V. cholerae (12; 7120)	6.9	5.5	4.8	5.5

The total number of proteins and amino acids on which the analyses were based is indicated in parentheses. Special features of the *C. salexigens* and the *Halobacterium* proteins as compared to the proteins of the non-halophilic bacteria are indicated in boldface

Table 3 'Halophilic' signatures of ribosomal proteins periplasmic binding proteins of ABC transporters and selected enzymes of the central metabolic pathways of *C. salexigens* as compared to *E. coli*, *P. aeruginosa*, *V. cholerae*, and *Halobacterium* NRC-1. Calculations were based on the same proteins featured in Table 2

	Acidic	Basic	Acidic/Basic	Ser + Thr	Hydrophobic
Ribosomes					
C. salexigens	**11.3**	17.6	0.64	**10.0**	35.1
E. coli	10.9	18.1	0.60	9.6	38.1
P. aeruginosa	10.9	18.1	0.61	9.8	36.2
V. cholerae	10.7	17.9	0.60	9.5	37.8
Halobacterium	**19.0**	**12.1**	**1.57**	**11.2**	**31.9**
Substrate binding proteins					
C. salexigens	**14.8**	**7.8**	**1.91**	11.6	37.6
E. coli	11.2	10.7	1.05	11.4	37.8
P. aeruginosa	11.6	11.5	1.01	10.1	38.4
V. cholerae	11.4	**9.7**	**1.17**	12.3	37.9
Enzymes of the central metabolism					
C. salexigens	**13.9**	10.3	**1.35**	9.9	37.2
E. coli	12.8	10.7	1.19	10.8	37.5
P. aeruginosa	12.8	10.9	1.17	9.8	38.4
V. cholerae	12.4	10.4	1.20	10.2	38.2

Acidic: Glu + Asp; Basic: Lys + Arg; Hydrophobic: Ala + Val + Leu + Ile + Phe + Met, not taking into account the N-terminal Met encoded by the initiation codon. The values are given in mole-percent of the total number of amino acid residues or as ratios, as appropriate. Special features of the *C. salexigens* and the *Halobacterium* proteins as compared to the proteins of the non-halophilic bacteria are indicated in boldface

content of the former would not cause a random skewing toward a higher Glu and Asp content in its periplasmic proteins. We also examined outer membrane proteins, and for these, the representatives from *C. salexigens* showed a tendency for the lowest pIs among the four bacteria, albeit the differences are less pronounced than for the soluble periplasmic proteins. However, we were able to recognize only a relatively few predicted outer membrane proteins in all four bacterial species, and therefore, the significance of this result is uncertain.

There are a few reports of the increased content of acidic amino acids in the proteins associated with the surface layers of halophilic microorganisms that exclude salt from their cytoplasm. Examination of the amino acid composition of the bulk protein and the membrane fraction of different species of *Halorhodospira* and *Ectothiorhodospira*—halophilic, in part also alkaliphilic, anoxygenic photosynthetic bacteria that produce glycine betaine, ectoine, and trehalose as osmotic solutes—showed little difference in the amino acid composition of the total cellular protein, but the membrane fraction of the most halophilic species showed a trend toward a more acidic nature of the proteins (Imhoff et al. 1983). Another interesting case is that of the acidic cytoplasmic membrane-bound transferrin and carbonic anhydrase of the halotolerant green alga *Dunaliella salina*, an organism that synthesizes glycerol as a compatible solute. The carbonic anhydrase (pI 4.6, as compared with 7.1–8.9 in other similar carbonic anhydrases) retains its activity and conformation over a large range of salinities (Fischer et al. 1996, 1997; Bageshwar et al. 2004).

A more detailed analysis of the amino acid composition of the different classes of proteins (Table 2) revealed that the differences in pI values of *C. salexigens* and non-halophilic bacteria can be ascribed to a general decrease in Lys residues in the former organism. The periplasmic substrate binding proteins are further enriched in both Asp and Glu. A slight increase in Asp but not in Glu was evident in the enzymes of the central metabolism. The slightly increased acidity of the ribosomal proteins appears to be due to a decreased Lys content (Table 2). The reason for the overall increased acidity of the *C. salexigens* proteins thus differs among the individual proteins, and no general trend can be observed.

An excess of acidic over basic amino acids is just one of the features of typically halophilic proteins. Other such characteristics are a decreased content of hydrophobic amino acids and an increased content of borderline hydrophobic amino acids Ser and Thr. The bulk protein of *Halomonas elongata*, a close relative of *Chromohalobacter*, does not show any of these additional properties (Gandbhir et al. 1995; Oren 1995). These additional halophilic signatures are also not present in the highly acidic periplasmic substrate binding proteins of *C. salexigens*. However, its ribosomal proteins do show a slightly increased content of Ser + Thr and a slightly decreased content of hydrophobic amino acids compared to the ribosomal proteins of the non-halophiles (Table 3).

The findings described above suggest that a major difference in the proteins of the moderate halophile *C. salexigens* and non-halophilic bacteria is found in the periplasmic proteins, exemplified by the substrate binding proteins of transport systems. The highly acidic nature of these proteins may enable them to function at high salt concentrations. The increased acidity reported in a major outer membrane porin of the *Chromohalobacter marismortui* as compared to the equivalent porin of related non-halophiles shows the same trend (Tokunaga et al. 2004). It is possible that the inherent salt requirement of such proteins determines the moderate to high concentrations of salt required for the growth of such halophiles. Further characterization of these binding proteins will be needed to test this hypothesis. Examination of the properties of the substrate binding proteins of related halotolerant organisms such as *Halomonas magadiensis* or *H. desiderata* (both moderate alkaliphiles), which show a far lower salt requirement while tolerating salt concentrations almost as high as *C. salexigens* (Ventosa et al. 1998), may provide further insights.

A complement of salt-tolerant substrate binding proteins is of course not the only special property in which a moderate halophile should differ from its non-halophilic counterparts. Other such properties are potent transport systems that regulate the intracellular ionic content of the cells and genes for the biosynthesis and/or transport of organic osmotic solutes. Such genes have been characterized in the past for the *Halomonas–Chromohalobacter* group (Cánovas et al. 1996, 1997, 1998), and additional ones may be identified in the *C. salexigens* genome.

Although one can rationalize why the periplasmic proteins of halophiles require special amino acid composition to be able to function in media of high salinity, why the cytoplasmic proteins of *C. salexigens* show a slight excess in acidic amino acids as compared to its non-halophilic counterparts is an open question. This may be related to the potentially increased ion concentration in such cells, as appears from many reports. However, it is possible that such apparently high ionic concentrations are at least in part due to artifacts during the experimental handling of the cells (Ventosa et al. 1998; Oren 2002). The salt sensitivity of protein synthesis by ribosomes of moderately halophilic bacteria suggests that their intracellular ion concentrations should indeed be low (Wydro et al. 1975, 1977). The possibility should therefore be envisaged that also the presence of molar concentrations of organic uncharged or zwitterionic compatible solutes may require some adaptation of the enzymes to allow optimal activity, even if such an adaptation is much less dramatic than in the case of *Halobacterium* which accumulates molar concentrations of KCl.

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