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ORIGINAL PAPER

Acid-shifted isoelectric point profiles of the proteins in a hypersaline microbial mat: an adaptation to life at high salt concentrations?

Rahel Elevi Bardavid · Aharon Oren

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Abstract In a metagenomic analysis of a stratified hypersaline (9 % salt) microbial mat in Guerrero Negro, Mexico, Kunin et al. (Mol Systems Biol 4:198, 2008) detected a significantly acid-shifted proteome, and concluded that adaptation by enriching proteins with acidic amino acids is more widespread than previously assumed. We here reevaluate these data and conclusions by comparing the isoelectric point profiles of the Guerrero Negro microbial mats (average isoelectric point 6.8) with those of the proteins encoded by the genomes of prokaryotes adapted to different salt concentrations ranges and belonging to different phylogenetic and physiological groups. Average isoelectric points below 6.8 were found not only in the proteomes of the moderately halophilic aerobic bacteria *Halomonas elongata* and *Chromohalobacter salexigens*, but even in common types of marine bacteria of the genera *Alteromonas* and *Aliivibrio*. We did not find clear evidence that the isoelectric point profile of the Guerrero Negro microbial mat can be considered to be the result of species-independent molecular convergence of the members of the microbial community determined by the salinity of the overlaying brine.

Keywords Halophilic · Hypersaline · Acidic proteins · Isoelectric point · Proteome

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R. Elevi Bardavid · A. Oren (✉)
Department of Plant and Environmental Sciences,
The Institute of Life Sciences, The Hebrew University
of Jerusalem, 91904 Jerusalem, Israel
e-mail: aharon.oren@mail.huji.ac.il

Introduction

Microorganisms living in saline and hypersaline environments need to balance their cytoplasm osmotically with their surrounding medium. The most common strategy used is the biosynthesis and/or accumulation of 'compatible' solutes—small organic molecules such as ectoine or glycine betaine. Intracellular salt concentrations are then kept at low levels, and no drastic modifications of most cellular proteins are needed (Galinski 1995; Oren 2006). A fundamentally different mode of osmotic adaptation is used by two groups of extremely halophilic aerobic heterotrophic prokaryotes: the Archaea of the family Halobacteriaceae and *Salinibacter* (Bacteroidetes) (Antón et al. 2002). Here molar concentrations of K^+ and Cl^- are accumulated intracellularly. In such cells the entire intracellular proteome must be adapted to function in saturated or near-saturated KCl solutions (Oren 2006).

It is known already for more than 40 years that the proteins of *Halobacterium* and other extremely halophilic Archaea show a large excess of acidic amino acids (glutamate, aspartate) over basic amino acids (lysine, arginine) (Reistad 1970). Their highly negatively charged proteins require high ionic concentrations for structural stability and activity (Lanyi 1974; Mevarech et al. 2000). *Salinibacter*, although phylogenetically unrelated, has a life style very similar to that of *Halobacterium*, and also shows a highly acidic proteome (Mongodin et al. 2005). On the other hand, there is no a priori reason why microorganisms that use organic osmotic solutes should possess an unusually large excess of acidic amino acids, with the possible exception of the periplasmic proteins and excreted enzymes that are exposed to the high salinity of the medium (Oren et al. 2005).

In view of the above, the finding by Kunin et al. (2008) of a significantly acid-shifted proteome in a stratified microbial mat growing at the relatively low salt concentration of 9 % in the lagoons of Guerrero Negro, Baja California Sur, Mexico, was unexpected. Halophilic Archaea of the order Halobacteriales and other known extremely halophilic ‘salt-in’ strategists cannot be expected to thrive at this salinity. Still, in spite of the strong phylogenetic variation between the communities in the different layers of the mat observed on a millimeter scale, as deduced from metagenomic data, all layers displayed near-identical and acid-shifted isoelectric point profiles. The average isoelectric point (6.8) of the proteins encoded by the metagenomes from the mat layer communities was conspicuously acid shifted when compared with most non-halophilic bacteria and microbiomes (Fig. 2A in Kunin et al. 2008). The authors concluded that adaptation by enriching proteins with acidic amino acids is more widespread than previously assumed, and they claimed their observation to be the first example of species-independent molecular convergence in a microbial community.

We here reevaluate the data and the conclusions presented by Kunin et al., comparing the isoelectric point profiles of the Guerrero Negro microbial mats with those of the proteins encoded by the genomes of prokaryotes adapted to different salt concentration ranges and belonging to different phylogenetic and physiological groups.

Methods

The following prokaryote genomes were included in our comparative analyses: the extremely halophilic archaeon *Halobacterium* NRC-1 (Euryarchaeota, Halobacteriales) (Ng et al. 2000); the extremely halophilic bacterium *Salinibacter ruber* M31^{T} (Bacteroidetes, Cytophagales) (Mongodin et al. 2005); the moderately halophilic bacterium *Halomonas elongata* 1H9^{T} (Gammaproteobacteria, Oceanospirillales) (Schwibbert et al. 2011); the moderately halophilic bacterium *Chromohalobacter salexigens* DSM 3043^{T} (Gammaproteobacteria, Oceanospirillales) (Oren et al. 2005); the marine bacterium *Alteromonas macleodii* ATCC 27126^{T} (Gammaproteobacteria, Alteromonadales); the marine bacterium *Aliivibrio fischeri* strain MJ11 (Gammaproteobacteria, “Vibrionales”) (Mandel et al. 2009); the marine bacterium “Pelagibacter ubique” (a name in violation of Rule 12c of the Bacteriological Code, representing the phylotype of SAR-11, abundant in the world oceans) (Alphaproteobacteria) (Giovannoni et al. 2005); the anaerobic fermentative halophile *Halanaerobium praevalens* GSL^{T} (Clostridia, Halanaerobiales) (Ivanova et al. 2011); the anaerobic fermentative halophile *Flexistipes sinusarabici* MAS 10^{T} (Deferribacteres, Deferribacterales), isolated from a deep-sea brine (Lapidus et al. 2011); and the anaerobic haloalkalithermophile *Natranaerobius thermophilus* JW.NM-WN-LF^{T} (Clostridia, Natranaerobiales) (Zhao et al. 2011). Accession numbers for these sequences are, respectively, NC_00260, CP000159, NC_014532, NC_007963, Gi03187, NC_011184, NC_007205, CP002175, CP002858, and CP001034-CP001036.

From the genome annotations we extracted those sequences encoding proteins or putative proteins, and calculated for each protein sequence the predicted 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

Evaluation of the p*I* profiles from the Guerrero Negro hypersaline microbial mat as compared to the extreme halophiles *Halobacterium* and *Salinibacter*

The average p*I* distribution of the proteins encoded by the metagenome from the different layers in the Guerrero Negro microbial mat found below brines of 9 % salinity is reproduced in Fig. 1. The figure shows a broad peak at p*I* values 4.5–4.9, with a considerable fraction of the proteins having p*I* values in the alkaline range. The average p*I* value calculated is 6.8 (Figs. 3A, 2A in Kunin et al. 2008, respectively). A similar p*I* distribution was found for the metagenome of a hypersaline (6 % salt) lagoon on the Galapagos Islands (Rusch et al. 2007; Ghai et al. 2011).

The major peak at p*I* values below 5 indeed indicates a majority of acidic proteins. However, the ‘halophilic’ character of the proteome is much less pronounced than

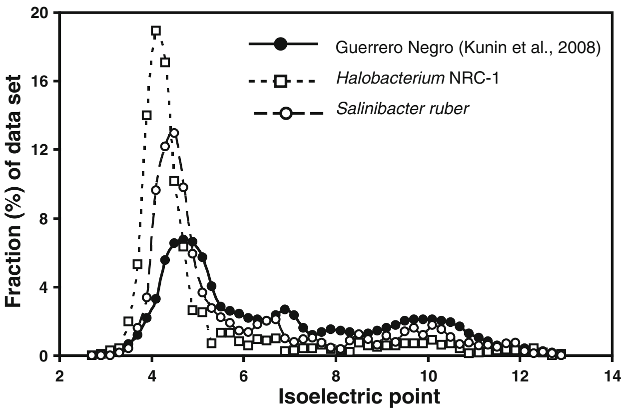

Fig. 1 Isoelectric point profiles, calculated at 0.2 intervals, of predicted proteins found in the metagenomic analysis of the hypersaline microbial mats of Guerrero Negro (from Kunin et al. 2008) and of proteins encoded by the genomes of the extreme halophilic archaeon *Halobacterium* NRC-1 (2,675 proteins; average p*I* 5.03) and the extreme halophilic bacterium *Salinibacter ruber* (2,845 proteins; average p*I* 5.92)

that of the proteins encoded by the genomes of *Halobacterium* NRC-1 and *S. ruber*, also presented in Fig. 1: there the peaks are located at p*I* values of 4.1 and 4.4–4.5, respectively, and there are much less proteins with p*I* values in the alkaline range, resulting in average p*I* values of 5.03 and 5.92, respectively (see also Mongodin et al. 2005). Patterns similar to those of *Halobacterium* and *Salinibacter* are typically found in the metagenomes of hypersaline environments with salt concentrations exceeding 25 % salt. The p*I* profile presented by Ghai et al. (2011) for the metagenome recovered from a Spanish saltern pond with 19 % salt already shows a similar trend, which can be explained by the abundance of Euryarchaeota already at that salinity (30 % of the 16S rRNA sequences recovered).

The p*I* profiles of moderate halophiles that exclude salt from their cytoplasm

It may be expected that moderately halophilic bacteria that synthesize and/or accumulate organic compatible solutes do not need far-going adaptations of the intracellular proteins to the presence of high salt concentrations. The prediction is thus that the proteome will not be unusually acidic. Indeed, in an earlier study it was shown that most of the selected proteins encoded by the genome of *C. salexigens* were 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). It should be noted that only 238 out of the 3,319 proteins encoded by the genome were included in the survey.

The two moderate halophiles included in our present study, *H. elongata* and *C. salexigens*, are Gammaproteobacteria that synthesize ectoine as compatible solute and accumulate glycine betaine when available in the medium. Both showed a large peak of acidic proteins (maximum at p*I* 4.4–5.0 and 4.5–5.1, respectively) in the isoelectric point profiles of the proteins predicted from their genomes (Fig. 2); the average p*I* values calculated were 6.32 and 6.60, lower than the value of 6.7 found for the Guerrero Negro microbial mat. Based on the earlier presented analysis (Oren et al. 2005), the slightly elevated average acidity of the proteins of these two bacteria may at least in part be due to the need for its periplasmic proteins to function also at high salt concentrations.

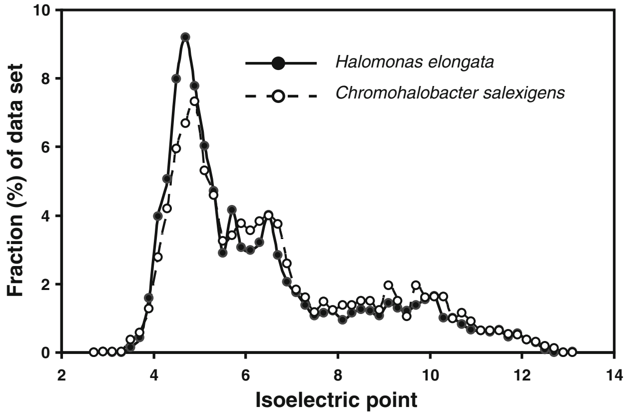

Fig. 2 Isoelectric point profiles, calculated at 0.2 intervals, of predicted proteins encoded by the genomes of the aerobic moderately halophilic bacteria *Halomonas elongata* (3,474 proteins; average p*I* 6.32) and *Chromohalobacter salexigens* (3,319 proteins; average p*I* 6.60)

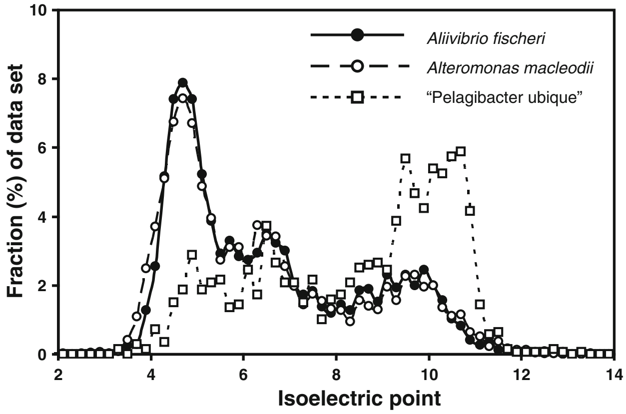

Fig. 3 Isoelectric point profiles, calculated at 0.2 intervals, of predicted proteins encoded by the genomes of the aerobic marine bacteria *Aliivibrio fischeri* (4,040 proteins; average p*I* 6.52), *Alteromonas macleodii* (4,396 proteins; average p*I* 6.46), and "Pelagibacter ubique" (1,393 proteins; average p*I* 8.42)

The p*I* profiles of heterotrophic marine bacteria

As the two moderately halophilic bacteria, adapted to grow at salt concentrations above those of seawater, showed a pronounced peak of acidic proteins in the p*I* profiles, we also included a number of typical marine bacteria in our comparative study. Figure 3 shows the p*I* profiles of two abundant marine Gammaproteobacteria: *Alteromonas macleodii*, a bacterium ubiquitous in the world's oceans (Ivars-Martínez et al. 2008) and the luminescent *Aliivibrio fischeri*. Both show a pronounced peak in the acidic range (maximum at p*I* values of 4.6–4.8), with average p*I* values of 6.46 and 6.52, respectively. If this pattern will prove to be characteristic of marine bacterioplankton, it may be noted that it is very similar to that of the Guerrero Negro microbial mats shown by Kunin et al. (2008).

However, not all marine bacteria share this pattern. A small bacterium belonging to the Alphaproteobacteria, provisionally named "Pelagibacter ubique" (the SAR-11 phylotype), probably representing the most abundant type of bacteria in the oceans, has been sequenced. Its small genome encodes for 1,393 proteins only, and these proteins show an excess of 2 mol % of basic amino acids (lysine + arginine) over acidic amino acids (glutamate + aspartate). For comparison, *Halobacterium* NRC-1, *S. ruber* and *H. elongata* have an excess of acidic amino acids of 7.5, 4.1, and 2.8 mol %, respectively (Elevi Bardavid and Oren 2012). Most of the proteins of "P. ubique" have p*I* values between 9.4 and 10.8, the average value being 8.42.

The biphasic p*I* distribution with peaks at p*I* 4.6 and 10.2, shown by Ghai et al. (2011) for the deep chlorophyll maximum in the Mediterranean Sea (3 % salt) can be explained by the simultaneous presence of well-known types of marine bacteria such as *Aliivibrio* and *Alteromonas* spp. (contributing the major peak in the acidic p*I* range) and "Pelagibacter ubique" with its alkaline proteome, found at an abundance of 35 % among the 16S rRNA gene sequences recovered (Ghai et al. 2010).

The proteome of anaerobic halophilic bacteria revisited

In a previous study, we assessed the nature of the proteins encoded by the genomes of three members of the order Halanaerobiales: *Halanaerobium praevalens*, "Halanaerobium hydrogeniformans", and *Halothermothrix orenii* (Elevi Bardavid and Oren 2012). Members of this order reportedly adjust the osmotic pressure of their cytoplasm with KCl and possess proteins that are active in the presence of high intracellular KCl concentrations. Unexpectedly, these anaerobes do not possess the typical acidic signature of 'halophilic' proteins of other 'salt-in' strategists such as the Archaea of the order Halobacteriales or of the extremely halophilic bacterium *Salinibacter*.

Recently the genomes of two other obligatory anaerobic halophiles, phylogenetically unrelated to the Halanaerobiales, have been sequenced: *Flexistipes sinusarabici*, isolated from a deep-sea brine pool on the bottom of the Red Sea (Fiala et al. 1990), and *Natranaerobius thermophilus*, an anaerobic halothermoalkaliphile from the Wadi An Natrun lakes, Egypt (Mesbah et al. 2007). *F. sinusarabici* belongs phylogenetically to the Deferribacteres, a deep branching group within the Bacteria; it grows at salt concentrations between 3 and 10 % and possibly higher as well. Nothing is known yet about its mode of osmotic adaptation. Its isoelectric point profile resembles that of *H. praevalens*: no pronounced acidic nature of the proteins and an average p*I* of 7.47, as compared to 7.42 calculated for *H. praevalens*. *N. thermophilus*, classified in a newly established order within the Clostridia, is markedly thermophilic (optimum temperature 53 °C) and alkaliphilic (optimum pH 9.5), and requires higher salt concentrations (3.1–4.9 M Na^+). It has a markedly acidic proteome, with an average p*I* of 6.27 (Fig. 4). Large variations in the p*I* profiles may thus also occur among the anaerobic halophilic bacteria.

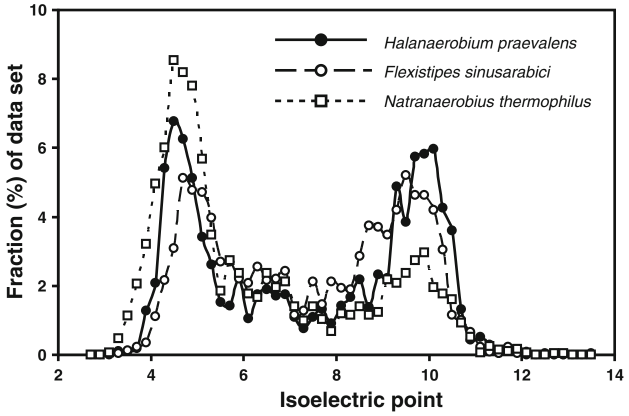

Fig. 4 Isoelectric point profiles, calculated at 0.2 intervals, of predicted proteins encoded by the genomes of the anaerobic halophiles *Halanaerobium praevalens* (2,110 proteins; average p*I* 7.42) (from Elevi Bardavid and Oren 2012), *Flexistipes sinusarabici* (2,261 proteins; average p*I* 7.47), and *Natranaerobius thermophilus* (2,906 proteins; average p*I* 6.27)

Final comments

Analysis of the proteins encoded by a number of representative genomes of marine bacteria and moderately halophilic aerobes does not support the conclusion by Kunin et al. (2008) that the average isoelectric point (6.8) of the proteins encoded by the metagenome of the different layers within the Guerrero Negro microbial mats found below brine of 9 % salt was conspicuously acid-shifted. Even lower average isoelectric points were found not only in the proteomes of moderately halophilic aerobic bacteria such as *H. elongata* and *C. salexigens*, but even in common types of marine bacteria of the genera *Alteromonas* and *Aliivibrio*. Thus, we did not find clear evidence that the isoelectric point profile of the Guerrero Negro microbial mat can be considered to be the result of species-independent molecular convergence of the members of the microbial community determined by the high (~2.5 times seawater) salinity of the overlaying brine. The proteomes of 'salt-in' strategist microorganisms—the members of the Halobacteriales (Archaea) and *Salinibacter* (Bacteria)—are much more acidic as appears from their far lower average p*I* values. The similar features of those organisms provide an excellent example of convergent evolution (Mongodin et al. 2005).

The data set collected by Kunin et al. (2008) was included in a comparative analysis by Rhodes et al. (2010) to assess the general trend of increased average protein acidity with increased salinity on an environmental scale. Figure 3 in Rhodes et al. (2010), based on calculations of the ratio of acidic to basic amino acids in the predicted proteins rather than on predicted p*I* values, shows a slightly increased ratio of acidic to basic amino acids in the Guerrero Negro mat as compared to marine ecosystems.

However, the values are much lower than those observed in the hypersaline Dead Sea, where the microbial community is dominated by halophilic Archaea of the order Halobacteriales. A more in-depth analysis of the metagenomic data may answer the question whether the slight increase in the ratio of acidic to basic amino acids in the hypersaline microbial mat may be due to a general slight preference for acidic amino acids in the proteins or to specific acidic proteins required for certain specialized functions in microorganisms living at elevated salt concentrations, such as the acidic periplasmic binding proteins identified in the genome of *C. salexigens* (Oren et al. 2005).

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