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RESEARCH ARTICLE

Net Charges of the Ribosomal Proteins of the *S10* and *spc* Clusters of Halophiles Are Inversely Related to the Degree of Halotolerance

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ABSTRACT Net positive charge(s) on ribosomal proteins (r-proteins) have been reported to influence the assembly and folding of ribosomes. A high percentage of r-proteins from extremely halophilic archaea are known to be acidic or even negatively charged. Those proteins that remain positively charged are typically far less positively charged. Here, the analysis is extended to non-archaeal halophilic bacteria, eukaryotes, and halotolerant archaea. The net charges (pH 7.4) of the r-proteins that comprise the *S10-spc* operon/cluster from individual microbial and eukaryotic genomes were estimated and intercompared. It was observed that, as a general rule, the net charges of individual proteins remained mostly basic as the salt tolerance of the bacterial strains increased from 5 to 15%. The most striking exceptions were the extremely halophilic bacterial strains, *Salinibacter ruber* SD01, *Acetohalobium arabaticum* DSM 5501 and *Selenihalanaerobacter shriftii* ATCC BAA-73, which are reported to require a minimum of 18% to 21% salt for their growth. All three strains have higher numbers of acidic *S10-spc* cluster r-proteins than what is seen in the moderate halophiles or the halotolerant strains. Of the individual proteins, only uL2 never became acidic. uS14 and uL16 also seldom became acidic. The net negative charges on several of the *S10-spc* cluster r-proteins are a feature generally shared by all extremely halophilic archaea and bacteria. The *S10-spc* cluster r-proteins of halophilic fungi and algae (eukaryotes) were exceptions: these were positively charged despite the halophilicity of the organisms.

IMPORTANCE The net charges (at pH 7.4) of the ribosomal proteins (r-proteins) that comprise the *S10-spc* cluster show an inverse relationship with the halophilicity/halotolerance levels in both bacteria and archaea. In non-halophilic bacteria, the *S10-spc* cluster r-proteins are generally basic (positively charged), while the rest of the proteomes in these strains are generally acidic. On the other hand, the whole proteomes of the extremely halophilic strains are overall negatively charged, including the *S10-spc* cluster r-proteins. Given that the distribution of charged residues in the ribosome exit tunnel influences cotranslational folding, the contrasting charges observed in the *S10-spc* cluster r-proteins have potential implications for the rate of passage of these proteins through the ribosomal exit tunnel. Furthermore, the universal protein uL2, which lies in the oldest part of the ribosome, is always positively charged irrespective of the strain/organism it belongs to. This has implications for its role in the prebiotic context.

KEYWORDS net charges, ribosomal proteins, *S10-spc* cluster, halophiles, *S10-spc* operons/cluster

The ribosome is a universal molecular machine comprised of RNA and proteins (1) which catalyzes coded protein synthesis in all three domains of life (2–4). Thirty-four ribosomal proteins (r-proteins) are universally conserved (5–9). Of these, 21 are encoded

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by two large clusters which are analogous to the *S10* and *spc* operons in *E. coli*. These clusters contain four additional genes in *Archaea* and *Eukarya* (9). Given that RNA is negatively charged, the electrostatic properties of r-properties are expected to play a role in stabilizing r-protein-rRNA interactions in the ribosome structure.

In previous examinations of the electrostatic properties of r-proteins, it was observed that extremely halophilic archaeal r-proteins were observed in general to be negatively charged. This is in stark contrast with those from non-halophilic *Archaea* (10, 11). The proteomes of halophilic species are overrepresented by acidic residues (12, 13). It is thought that this may reflect a genetic adaptation. Earlier work by Kushner (1978) (14), Kushner and Kamekura (1988) (15), and Ventosa et al. (1998) (16), classified halophiles based on their salt requirement and tolerance limit(s). Moderate halophiles have been classified as those growing optimally between 0.5 M and 2.5 M salt (14, 15). Strains that can tolerate a broad range of low-high salt concentrations are classified as halotolerant. When growth is possible from low concentration and extends above 2.5 M, these strains are classified as extremely halotolerant. Halophiles that require at least 2 M salt for growth are considered extreme halophiles (15).

Despite the availability of cultured bacteria, archaea, fungi, and algae, and their characterized genomes/proteomes (16–32), little is known of the electrostatic properties of the r-proteins of halophilic and halotolerant bacteria, fungi, algae, and moderately halophilic archaea. These halophiles range from moderately halophilic, halotolerant, and extremely halotolerant to extremely obligately halophilic bacteria, fungi, and algae (16, 17, 19, 33–67). Additionally, there are several moderately halophilic archaea, including several methanogens (20, 39, 68, 69). On the other hand, extremely halophilic strains tend to be obligately halophilic, with minimum salt requirements of 18% to 21% (70). *A. arabaticum* (18, 71, 72), *S. shriftii* (73) and *S. ruber* (74), with salt requirements of 15% to 18% (71), 21% (73), and 20% to 25% (75), respectively (Table S1), are examples of extremely halophilic bacteria.

Here, the results of comparison of the net charges (pH 7.4) of *S10-spc* r-protein homologs from several halotolerant, extremely halophilic, and non-halophilic microbial (bacterial and archaeal) and eukaryotic genomes are reported. The results in part correlate with the extent of halotolerance.

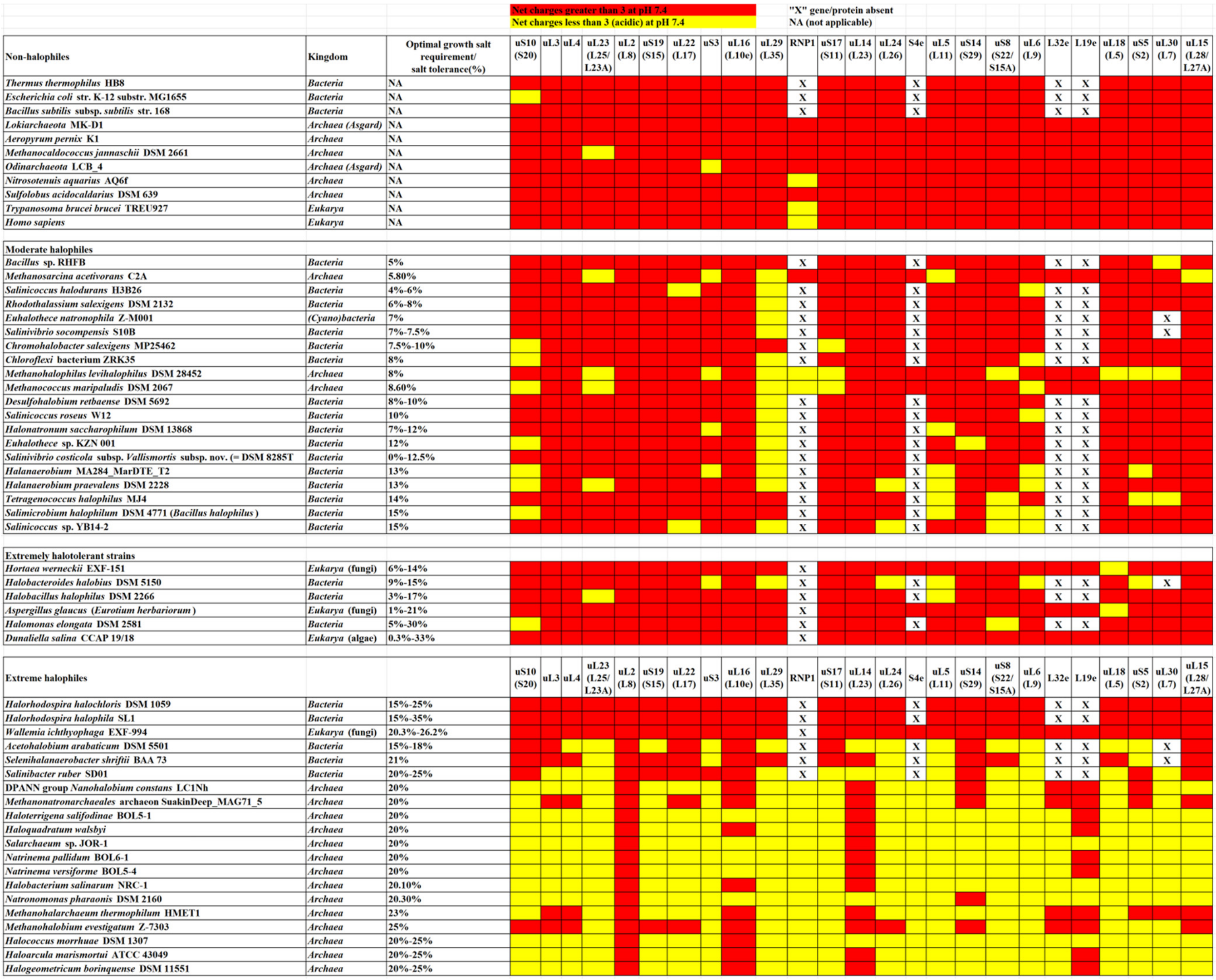
RESULTS

Non-halophiles, including Archaea and Eukaryotes. The charges on the r-proteins of the homolog equivalents of the *S10-spc* operon/cluster were examined and compared to each other in the data set of protein sequences from bacteria, archaea, and eukaryotes. In both bacteria and archaea, an increase in the salt tolerance limit is inversely related to the net charges on the r-proteins examined (Fig. 1–4). Based on the general principle that weak acidity ranges from pH 3 to 6 and strong acidity is pH $<$3 (76, 77), the cutoff pH for acidity of the charges was set at 3. As the level of tolerance/halophilicity goes above 15%, many of the r-proteins show charges that are less than 3.

Extremely halophilic archaea. In both the extremely halophilic bacteria and archaea examined, there is a significant increase in the number of *S10-spc* cluster r-proteins that are negatively charged or have charges of less than 3. Three extremely halophilic bacterial strains *Salinibacter ruber* SD01, *Acetohalobium arabaticum* DSM 5501, and *Selenihalanaerobacter shriftii* BAA-73 were part of this analysis (Fig. 3). Twelve of the 21 *S10-spc* cluster r-proteins in the strains *S. ruber* SD01 and *A. arabaticum* DSM 5501, and 6 of these proteins in the strain *S. shriftii* ATCC BAA-73, possess charges of less than 3 (acidic). The acidic properties of the *S10-spc* cluster r-proteins are shared by the halophilic archaea (Fig. 1).

Halotolerant/halophilic fungi (eukarya) and some halotolerant bacteria are exceptions to this. The bacterial strain *H. elongata* DSM 2581, which is tolerant to a broad range of salt concentrations (5% to 30%), is extremely halotolerant (78–80). The extremely halophilic bacterial strains *Halorhodospira halochloris* DSM 1059 and *Halorhodospira halophila* SL1 grow optimally at salt concentrations of 15% to 25% and 15% to 35%, respectively (81–84). In these three bacterial strains, all the *S10-spc* cluster r-proteins had charges that were greater than 3, except for uS10 and uS8 in *H. elongata* DSM 2581 (Fig. 1).

The extremely halotolerant fungal strains, namely *Aspergillus glaucus* (*Eurotium herbariorum*) and *Hortaea werneckii* EXF-151, the extremely halophilic fungal strain



🟥 Net charges greater than 3 at pH 7.4
🟨 Net charges less than 3 (acidic) at pH 7.4
"X" gene/protein absent
NA (not applicable)

Non-halophiles	Kingdom	Optimal growth salt requirement/ salt tolerance(%)	uS10 (S20)	uL3	uL4	uL23 (L25/ L23A)	uL2 (L8)	uS19 (S15)	uL22 (L17)	uS3	uL16 (L10e)	uL29 (L35)	RNP1	uS17 (S11)	uL14 (L23)	uL24 (L26)	S4e	uL5 (L11)	uS14 (S29)	uS8 (S22/ S15A)	uL6 (L9)	L32e	L19e	uL18 (L5)	uS5 (S2)	uL30 (L7)	uL15 (L28/ L27A)
Thermus thermophilus HB8	*Bacteria*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Escherichia coli str. K-12 substr. MG1655	*Bacteria*	NA	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Bacillus subtilis subsp. *subtilis* str. 168	*Bacteria*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Lokiarchaeota MK-D1	*Archaea (Asgard)*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Aeropyrum pernix K1	*Archaea*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Methanocaldococcus jannaschii DSM 2661	*Archaea*	NA	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Odinarchaeota LCB_4	*Archaea (Asgard)*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Nitrosotenuis aquarius AQ6f	*Archaea*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Sulfolobus acidocaldarius DSM 639	*Archaea*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Trypanosoma brucei brucei TREU927	*Eukarya*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Homo sapiens	*Eukarya*	NA	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Moderate halophiles																											
Bacillus sp. RHFB	*Bacteria*	5%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟨	🟥
Methanosarcina acetivorans C2A	*Archaea*	5.80%	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟨	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨
Salinicoccus halodurans H3B26	*Bacteria*	4%-6%	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟨	X	X	🟥	🟥	🟥	🟥
Rhodothalassium salexigens DSM 2132	*Bacteria*	6%-8%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Euhalothece natronophila Z-M001	*(Cyano)bacteria*	7%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	X	🟥
Salinivibrio socompensis S10B	*Bacteria*	7%-7.5%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	X	🟥
Chromohalobacter salexigens MP25462	*Bacteria*	7.5%-10%	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟨	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Chloroflexi bacterium ZRK35	*Bacteria*	8%	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟨	X	X	🟥	🟥	🟥	🟥
Methanohalophilus levihalophilus DSM 28452	*Archaea*	8%	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟨	🟥	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟨	🟨	🟨	🟥
Methanococcus maripaludis DSM 2067	*Archaea*	8.60%	🟨	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥
Desulfohalobium retbaense DSM 5692	*Bacteria*	8%-10%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Salinicoccus roseus W12	*Bacteria*	10%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟨	X	X	🟥	🟥	🟥	🟥
Halonatronum saccharophilum DSM 13868	*Bacteria*	7%-12%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟨	X	🟥	🟥	🟥	X	🟨	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Euhalothece sp. KZN 001	*Bacteria*	12%	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟨	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Salinivibrio costicola subsp. *Vallismortis* subsp. nov. (= DSM 8285T	*Bacteria*	0%-12.5%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Halanaerobium MA284_MarDTE_T2	*Bacteria*	13%	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟨	X	🟥	🟥	🟥	X	🟨	🟥	🟥	🟨	X	X	🟥	🟨	🟥	🟥
Halanaerobium praevalens DSM 2228	*Bacteria*	13%	🟨	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟨	X	🟨	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Tetragenococcus halophilus MJ4	*Bacteria*	14%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟨	🟥	🟨	🟥	X	X	🟥	🟨	🟨	🟥
Salimicrobium halophilum DSM 4771 (*Bacillus halophilus*)	*Bacteria*	15%	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	X	🟥	🟥	🟥	X	🟨	🟥	🟨	🟨	X	X	🟥	🟥	🟥	🟥
Salinicoccus sp. YB14-2	*Bacteria*	15%	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟨	X	🟥	🟥	🟨	X	🟥	🟥	🟨	🟨	X	X	🟥	🟥	🟥	🟥
Extremely halotolerant strains																											
Hortaea werneckii EXF-151	*Eukarya* (fungi)	6%-14%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥
Halobacteroides halobius DSM 5150	*Bacteria*	9%-15%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟨	X	🟥	🟥	🟨	X	🟨	🟥	🟥	🟨	X	X	🟥	🟨	X	🟥
Halobacillus halophilus DSM 2266	*Bacteria*	3%-17%	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟨	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Aspergillus glaucus (*Eurotium herbariorum*)	*Eukarya* (fungi)	1%-21%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟨	🟥	🟥	🟥
Halomonas elongata DSM 2581	*Bacteria*	5%-30%	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟥	🟥	🟨	🟥	X	X	🟥	🟥	🟥	🟥
Dunaliella salina CCAP 19/18	*Eukarya* (algae)	0.3%-33%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Extreme halophiles			uS10 (S20)	uL3	uL4	uL23 (L25/ L23A)	uL2 (L8)	uS19 (S15)	uL22 (L17)	uS3	uL16 (L10e)	uL29 (L35)	RNP1	uS17 (S11)	uL14 (L23)	uL24 (L26)	S4e	uL5 (L11)	uS14 (S29)	uS8 (S22/ S15A)	uL6 (L9)	L32e	L19e	uL18 (L5)	uS5 (S2)	uL30 (L7)	uL15 (L28/ L27A)
Halorhodospira halochloris DSM 1059	*Bacteria*	15%-25%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Halorhodospira halophila SL1	*Bacteria*	15%-35%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	X	X	🟥	🟥	🟥	🟥
Wallemia ichthyophaga EXF-994	*Eukarya* (fungi)	20.3%-26.2%	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	X	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Acetohalobium arabaticum DSM 5501	*Bacteria*	15%-18%	🟥	🟥	🟨	🟨	🟥	🟨	🟥	🟨	🟥	🟨	X	🟥	🟨	🟨	X	🟨	🟥	🟨	🟨	X	X	🟨	🟨	X	🟥
Selenihalanaerobacter shriftii BAA 73	*Bacteria*	21%	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟨	🟥	🟥	X	🟥	🟥	🟨	X	🟨	🟥	🟥	🟨	X	X	🟥	🟨	X	🟥
Salinibacter ruber SD01	*Bacteria*	20%-25%	🟥	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟨	X	🟨	🟨	🟨	X	🟨	🟥	🟨	🟨	X	X	🟨	🟥	🟨	🟥
DPANN group *Nanohalobium constans* LC1Nh	*Archaea*	20%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟥	🟥	🟨	🟥	🟨	🟨
Methanonatronarchaeales archaeon SuakinDeep_MAG71_5	*Archaea*	20%	🟨	🟥	🟥	🟨	🟥	🟨	🟥	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟥	🟥	🟨	🟥	🟨	🟥
Haloterrigena salifodinae BOL5-1	*Archaea*	20%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨
Haloquadratum walsbyi	*Archaea*	20%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨
Salarchaeum sp. JOR-1	*Archaea*	20%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨
Natrinema pallidum BOL6-1	*Archaea*	20%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨
Natrinema versiforme BOL5-4	*Archaea*	20%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨
Halobacterium salinarum NRC-1	*Archaea*	20.10%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨
Natronomonas pharaonis DSM 2160	*Archaea*	20.30%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨
Methanohalarchaeum thermophilum HMET1	*Archaea*	23%	🟨	🟥	🟥	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟥	🟨	🟥	🟥	🟥
Methanohalobium evestigatum Z-7303	*Archaea*	25%	🟥	🟥	🟥	🟨	🟥	🟥	🟥	🟨	🟥	🟨	🟨	🟨	🟥	🟥	🟨	🟨	🟥	🟨	🟨	🟥	🟥	🟨	🟨	🟨	🟥
Halococcus morrhuae DSM 1307	*Archaea*	20%-25%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨
Haloarcula marismortui ATCC 43049	*Archaea*	20%-25%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨
Halogeometricum borinquense DSM 11551	*Archaea*	20%-25%	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟨	🟨	🟨	🟨

FIG 1 Net charges of the ribosomal proteins of the *S10-spc* cluster from representative strains of *Bacteria*, *Archaea*, and *Eukarya*. Proteins that have net charges (at pH 7.4) greater than 3 are shown in red, while those with net charges lesser than 3 are in yellow.

W. ichthyophaga EXF-994, and the extremely halotolerant algae *Dunaliella salina*, were all isolated from hypersaline environments (34, 55, 56, 65, 85–91). Except for uL18 in *Aspergillus glaucus* and *Hortaea werneckii* EXF-151,the net charges (pH 7.4) of the r-proteins of the *S10-spc* clusters in these organisms were all greater than 3 (Fig. 1 and Fig. S1 to S2).

The r-proteins of the *S10-spc* operon cluster in non-halophilic bacteria, non-halophilic archaea, and eukarya had positive charges (>3) (Fig. 1). The exceptions to this were the r-proteins uS10 (in the bacterium *E. coli* MG1655), uL23 (in archaeon *M. jannaschii* DSM 2661), uS3 (in the Asgard archaeon *Odinarchaeota* LCB_4), uL16 (L10e; in the eukaryote *T. brucei brucei* TREU927) and RNase P1 (RNP1 in the two eukaryotes examined), which had charges of less than 3 (Fig. 1).

Within the set of r-proteins of the *S10-spc* operon clusters examined, r-protein uL2 was uniquely positively charged (>3) irrespective of the species or the domain to which the species belonged. In bacteria, uL2 homologs had the highest charges compared to other cluster proteins. In bacteria, the charges of the uL2 homologs were consistently higher than those of the other proteins in the *S10-spc* cluster. The uL2 homolog with the lowest net charge of 5 (at pH 7.4) was found in the extremely halophilic archaeon *H. salinarum* NRC-1 (Table S2). This was also considered when setting the cutoff value for the net charge(s) of the proteins examined. Likewise,

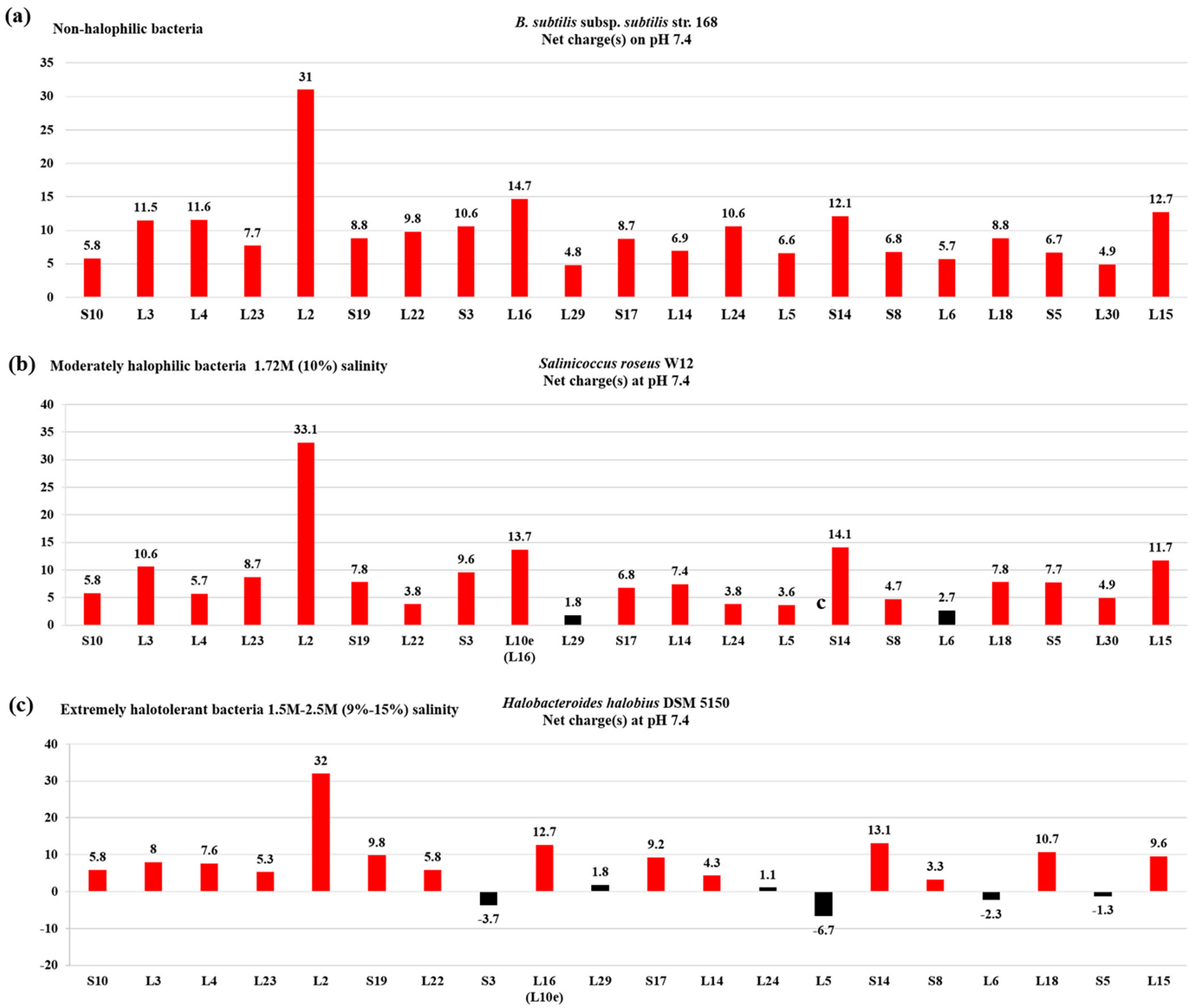


FIG 2 Net charges of the ribosomal proteins of the *S10-spc* cluster from representative strains of moderately halophilic Bacteria. (a) *B. subtilis* subsp. *subtilis* str. 168 (non-halophile). (b) *Salinicoccus roseus* W12 (10% salt). (c) *Halobacteroides halobius* DSM 5150 (9% to 15% salt). The charge value of each protein is shown for each bar; charges greater or lesser than 3 are shown in red and black, respectively.

most uL14 homologs showed charges of >3, except for the homologs in the extreme halophiles *A. arabaticum* DSM 5501 (Bacteria), *S. ruber* SD01 (Bacteria), *N. pharaonis* DSM 2160 (Archaea), and *H. morrhuae* DSM 1307 (Archaea) (Fig. 1 and 2).

Net charges on the non-r-proteins (*S10-spc* cluster). The whole proteomes of the extremely halophilic strains were overall negatively charged, including the r-proteins. In contrast, an unusual pattern was observed in the proteomes of the non-halotolerant bacteria, such as *E. coli* and *T. thermophilus*, and of the moderately halophilic *Bacillus* sp. RHFB (92), *E. natronophila* Z-M001 (cyanobacteria) (41), and *S. roseus* (46). In these strains, while the *S10-spc* cluster proteins showed net positive charges (Fig. 1), the rest of the proteome showed negative charges (data not shown).

DISCUSSION

Salt tolerance and net charges of r-proteins. In a previous analysis of the electrostatic properties of r-proteins from bacteria (*E. coli*, *T. thermophilus*, and *D. radiodurans*), halophilic archaea, and non-halophilic archaea, negative charges were uniquely found among the r-proteins of extremely halophilic archaea (10).

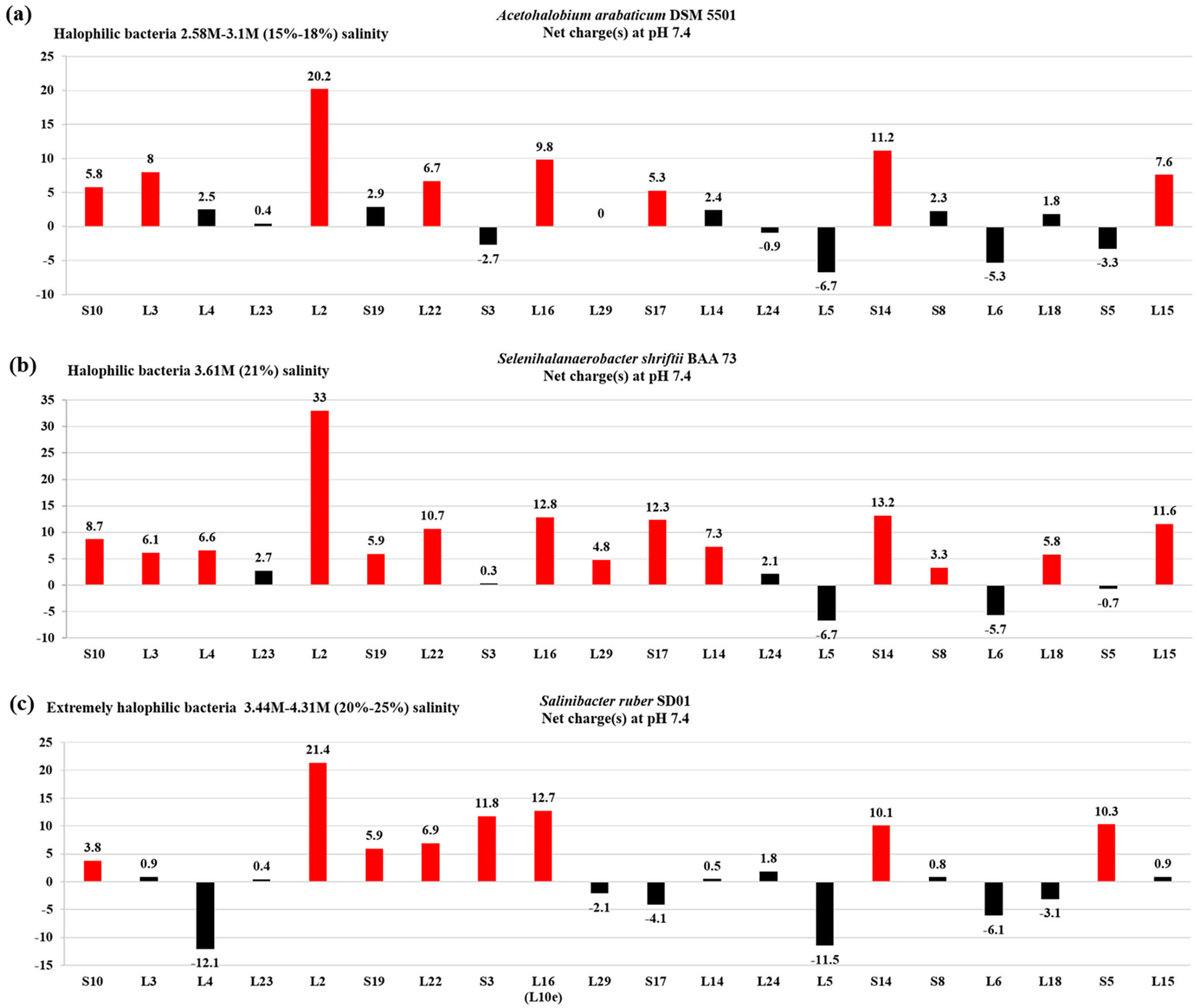


FIG 3 Net charges of the ribosomal proteins of the *S10-spc* cluster from representative strains of extremely halophilic bacteria. (a) *Acetohalobium arabaticum* DSM 5501 (15% to 18% salt). (b) *Selenihalanaerobacter shriftii* ATCC BAA-73 (21% salt). (c) *Salinibacter ruber* SD01 (20% to 25% salt). The charge value of each protein is shown for each bar; charges greater or lesser than 3 are shown in red and black, respectively.

In this study, an inverse relationship was observed between the halotolerance limits of bacteria/archaea and the net charges of r-proteins of the highly conserved *S10-spc* cluster. In the moderately halophilic bacteria or archaea, which can tolerate up to 15% salt concentration, the charges on the *S10-spc* cluster r-proteins are less than those of their homologs in non-halotolerant bacterial strains. Halotolerance is most likely an outcome of properties such as the production of intracellular osmolytes, solutes, or salting-out strategies (70, 86, 93) to counterbalance the ionic imbalance in fluctuating ionic environments. However, as halotolerance extends above 15%, many of the *S10-spc* cluster r-proteins of extremely halophilic bacterial and archaeal strains show net negative charges, implying a genomic-level adaptation that is unique to these strains (Fig. 1 and 2). In fact, it has been suggested that the halophilic bacterial strain *Salinibacter ruber* SD01 is similar to the extremely halophilic archaeal strains *Halobacterium salinarum* and *Haloarcula marismortui*, both at the genomic and at the physiological level (94). This gene-level adaptation strategy is most likely an evolutionary outcome to minimize the energy expenditure required to survive in very high salt concentrations.

In contrast to the drastic changes in the net charges of the r-proteins of the *S10-spc* cluster in extremely halophilic bacteria and archaea, the homologs of this cluster in

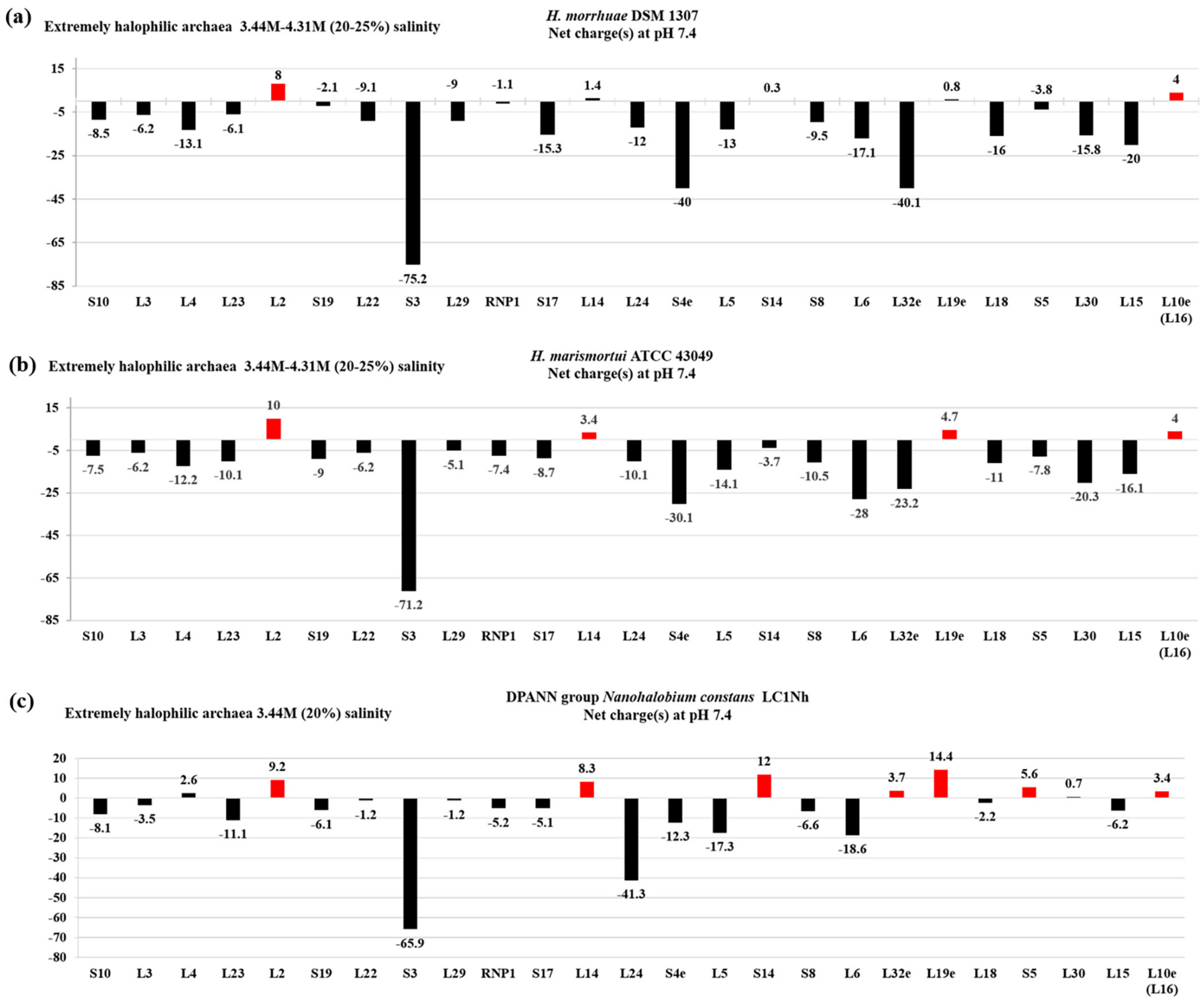


FIG 4 Net charges of the ribosomal proteins of the *S10-spc* cluster from representative strains of extremely halophilic Archaea. (a) *H. morrhuae* DSM 1307 (20% to 25% salt). (b) *H. marismortui* ATCC 43049 (20% to 25% salt). (c) DPANN group *Nanohalobium constans* LC1Nh (20% salt). The charge value of each protein is shown for each bar; charges greater or lesser than 3 are in red and black, respectively.

halophilic fungi and algae (eukaryotes) show positive charges (Fig. 1, Fig. S1 to S2). In an earlier study, it was observed that high levels of acidic residues were frequent in the protein families of three extremely halotolerant/halophilic fungal species: *W. ichthyophaga*, *H. werneckii*, and *E. rubrum* (95). Such strains have also been reported to use traits such as melanin-like pigment production, compatible solute production, ion efflux mechanisms, morphological changes, and regulation of plasma membrane fluidity to survive in hypersaline conditions (86, 96–99). These observations in halophilic eukaryotes suggest that adaptation to hypersaline conditions is likely a result of a combination of acidic residues in proteins, as well as changes in physiology and biochemistry. This is clearly not the case with the *S10-spc* cluster protein homologs of the halophilic eukaryotic strains examined in this study.

Of all the *S10-spc* cluster r-proteins, the net charge on uL2 always remains >3, irrespective of the organism/domain, despite a steady decrease in the charge(s) corresponding with an increase in halotolerance (Table S2). uL2 is a universal r-protein and is among the first set of large subunit proteins to be assembled into the ribosome (6, 100, 101). In the assembled ribosome structure, it is in very close proximity to what is considered the oldest part of the ribosome, namely, the Peptidyl Transferase Center

(PTC) (4, 102, 103). It is also thought to be ancient in origin (104). The universality of the positive charge on all uL2 homologs has implications for the possible nature of its predecessor peptide in the prebiotic world. In the prebiotic scenario, a positively charged uL2 would have helped maximize stable adhesion/binding to the region surrounding the proto-PTC.

Implications of net charges on translational rate(s) and ribosome stability. It has been posited that adaptation to extreme environments, such as high salt, might involve structural alterations of proteins that do not affect their functions (105). The ribosome exit tunnel regulates translation and protein folding (106–108). Certain amino acid sequence segments are also known to stall ribosomes (109, 110). In a study on the proteomes of multiple organisms by Requião R.D. et al. (111), negatively charged proteins were found to be overrepresented. Thus, it was suggested that the charges on the nascent peptide are probably among the factors regulating translation efficiency and protein expression.

However, the vast differences in the net charges of many r-proteins of the *S10-spc* cluster between the non-halophiles, moderate halophiles, and extreme halophiles do not appear to significantly affect the ribosome tertiary structure. The core structure of the ribosome is shared by all three domains of life (5).

Furthermore, in studies on halophilic archaea, ribosome stability is known to be severely affected in low-salt concentration buffers (112, 113). However, despite the salt requirement for its stability, the overall basic structure of the ribosome in extreme halophilic archaea, such as *H. morrhuae* (113) or *H. marismortui* (114, 115), is similar to that found in bacteria. The amino acid residues are reported to undergo significant intermolecular segregation along the ribosomal proteins based on their charges. This has been shown to occur in such a manner as to have positively and negatively charged residues in buried and solvent export regions, respectively (10). With that said, the net low positive or negative charges of halophilic r-proteins found in extremely halophilic archaea/bacteria could be a major factor contributing to the high salt requirement for the stability of these ribosomes.

Finally, the distribution of charged residues in the ribosome exit tunnel (116) influences the landscape of cotranslational folding. For example, the positive charge density of r-proteins in *E. coli* is hypothesized to play a role in the cotranslational assembly of ribosomes by delaying the release of nascent r-proteins (117). Electrostatic interactions between positively charged residues (on the nascent peptide that is being synthesized) and the ribosomal tunnel are known to decrease translation rate (118). Therefore, the question is moot as to how negatively charged r-proteins of the *S10-spc* cluster in extreme halophiles affect the rate of the passage of the same through the negatively charged exit tunnel of the ribosome.

CONCLUSIONS

The ribosome in general, and the exit tunnel in particular, are primarily negatively charged due to an RNA component which favors positively charged ribosomal proteins. Herein, the charges of the highly conserved ribosomal proteins found in the *S10-spc* gene cluster were used to characterize the extent of halotolerance in various microorganisms. It was expected and found that bacteria such as *E. coli* or *B. subtilis* that have little to no salt tolerance have the most positively charged proteins. Thus, the charges of the r-proteins of such bacteria are not markedly different from those of other non-halophilic archaea/eukarya. However, an increase in the salt tolerance limit results in a shift toward a more permanent change in the genome, resulting in encoding r-proteins with lower charges. This is evident in the net charge profiles of bacteria capable of growing optimally at salt concentrations above 15%. This trend is similar to that observed in extremely halophilic archaea. Individual proteins behave differently, with uL2 always remaining positive, which may reflect its role in holding the two subunits together. Contrasting charges on the r-proteins in bacteria/archaea may have implications for the passage of the growing protein through the exit tunnel and thus for the translation rate.

MATERIALS AND METHODS

Protein and gene sequences from individual microbial (bacterial and archaeal) and eukaryotic organisms were downloaded from the public databases of the National Center for Biotechnology Information (NCBI) (119, 120). The net charges of all proteins (at pH 7.4) from each organism were estimated/calculated using the Isoelectric Point Calculator (121). The results were cross-verified with Prot Pi (https://www.protpi.ch/Calculator/ProteinTool) and Protein Calculator v. 3.4 (http://protcalc.sourceforge.net/). Additionally, the net charges (at pH 7.4) on the sequences of the r-proteins belonging to the equivalent of the *S10-spc* cluster from each genome were compared with those of the rest of the proteins in each genome.

Bacterial, archaeal strains and eukaryotes used in comparisons. A list of the organisms (and genomes) covered is given in Table S1 in the supplemental material.

Data availability. The data sets used and analyzed within the current study are available from the NCBI website as referenced in the paper.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.03 MB.

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M.R.T. and G.E.F. conceived and designed the study. M.R.T., D.A.B., and S.R. obtained the sequences, estimated the charges on the same and prepared the tables. M.R.T. prepared the figures. M.R.T. and G.E.F. prepared the manuscript paper, which was finalized with help from all authors. All authors read and approved the final manuscript. S.R. is a 11th grade student at Clements High School (Class of 2023) who volunteered with the group of George E. Fox at the University of Houston.

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