Chapter

Halocins, Bacteriocin-Like Antimicrobials Produced by the Archaeal Domain: Occurrence and Phylogenetic Diversity in *Halobacteriales*

Afef Najjari, Hiba Mejri, Marwa Jabbari, Haitham Sghaier, Ameur Cherif and Hadda-Imene Ouzari

Abstract

Members of extremely halophilic archaea, currently consisting of more than 56 genera and 216 species, are known to produce their specific bacteriocin-like peptides and proteins called halocins, synthesized by the ribosomal pathway. Halocins are diverse in size, consisting of proteins as large as 35 kDa and peptide "microhalocins" as small as 3.6 kDa. Today, about fifteen halocins have been described and only three genes, halC8, halS8 and halH4, coding C8, S8 and H4 halocins respectively have been identified. In this study, a total of 1858 of complete and nearly complete genome sequences of Halobacteria class members were retrieved from the IMG and Genbank databases and then screened for halocin encoding gene content, based on the BLASTP algorithm. A total of 61 amino acid sequences belonging to three halocins classes (C8, HalH4 and S8) were identified within 15 genera with the abundance of C8 class. Phylogenetic analysis based on amino acids sequences showed a clear segregation of the three halocins classes. Halocin S8 was phylogenetically more close to HalH4. No clear segregation on species and genera levels was observed based on halocin C8 analysiscontrary to HalH4 based analysis. Collectively, these results give an overview on halocins diversity within halophilic archaea which can open new research topics that will shed light on halocins as marker for haloarchaeal phylogentic delineation.

Keywords: archaea, bioinformatics, diversity, halocins, phylogeny

1. Introduction

Microorganisms of the third domain of life, Archaea, have been cultivated and described for more than 100 years [1], however, they have been first assigned to the Bacteria domain because of their great phenotypic similarities. In the late 1970s, Carl Woese and his collaborators, recognized the Archaea as the third domain of life on earth based on molecular phylogenetic analyses [2]. The dichotomous (eukaryotic/prokaryotic) classification was no longer valid, leading to a reclassification of

organisms as three separate domains: Eucarya (*Eukaryotes*), Archaea and Bacteria (bacteria) [3]. Archaea share several similarities with the other two domains of life. They are similar to size-level bacteria, organization of their chromosomes, absence of nucleus and organelles, presence of polycistronic transcription units and use of Shine-Dalgarno sequences for the initiation of the translation. In addition, it was shown that their metabolic proteins are essentially bacterial in nature following analysis of many complete genomes of Archaea [2]. Archaea also share similarities with the Eucarya domain, such as the proteins involved in key informational processes such as replication [4], transcription, translation [5, 6], DNA repair [7, 8], mRNA degradation and proteolysis. Translation in Archaea has eukaryotic initiation and elongation factors, and their transcription involves TATA binding protein and TFIIB [9].

The biotopes colonized by these microorganisms, are supposed to approach to the primitive terrestrial atmosphere (high salinity or pH, devoid of O_2, rich in H_2 and CO_2 constituting the raw materials for the production of methane) [10]. They present spectacular adaptations, especially in extreme environments. We distinguish: (i) Thermophilic Archaea: living at high temperatures (60–80°C) (ii) Hyperthermophilic Archaea: living at very high temperatures (up to 121°C); (iii) Psychrophilic Archaea: prefering low temperatures (below 15°C) [11]; (iv) Halophilic Archaea: colonizing very saline environments (3–5 M NaCl) such as the Dead Sea [12, 13]; (v) Acidophilic Archaea: living at low pH (as low as pH 1 and dying at pH 7) and Alkaliphilic Archaea: thriving at high pH (up to 9) [14].

2. Taxonomy of the archaeal domain

The first phylogenetic study based on the comparison of the 16S rDNA gene sequences coding for the small subunit, separated the first founding members of Archaea into two taxa, one grouping methanogenic species and those living under conditions of extreme salinity, the other containing species living at very high temperatures and at acidic pH [15]. Ten years later, analyses on a larger taxonomic group led to the division of the Archaea kingdom into two groups: (i) *Crenarchaeota*, which is composed exclusively of microorganisms living at very high temperatures, and (ii) *Euryarchaeota*, a heterogeneous group of species with different phenotypes (methanogenic species, species living at very high temperatures, moderate temperatures or at high salt concentrations) [3]. Fifteen years later and thanks to metagenomic analyses, two phyla, *Thaumarchaeota* and *Korarchaeota* were established based on the results of genomic comparison of two uncultivable strains, *Candidatus cenarchaeum symbosium* and *Candidatus korarchaeum cryptofilum*, with genomic traits belonging to both phyla *Crenarchaeota* and *Euryarchaeota* [16]. On the other hand, the symbiont *Nanoarchaeum equitans*, occupying cells of the host *Ignicoccus hospitalis*, showed even more genomic divergence with the other members of the *Crenarchaeota* and was therefore the first member of the phyla *Nanoarchaeota*.

Today, we count more of 15 phyla in the reign of Archaea, some of them having been grouped in superphylum. One distinguishes the superphylum TACK, proposed in 2011 and of which the eukaryotes would have evolved according to the theory of the eocyte, grouping *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota* and *Korarchaeota* phyla [17]. This superphylum has been joined by recently proposed phyla: *Bathyarchaeota*, *Geoarchaeota* and *Lokiarchaeota* [18]. Another superphylum, DPANN, was proposed in 2013 and includes *Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanoarchaeota* and *Nanohaloarchaeota* phyla (**Figure 1**) [19]. Finally, the phyla *Woesearchaeota* and *Pacearchaeota*, described in 2016, were grouped in the DPANN superphylum (**Figure 1**).

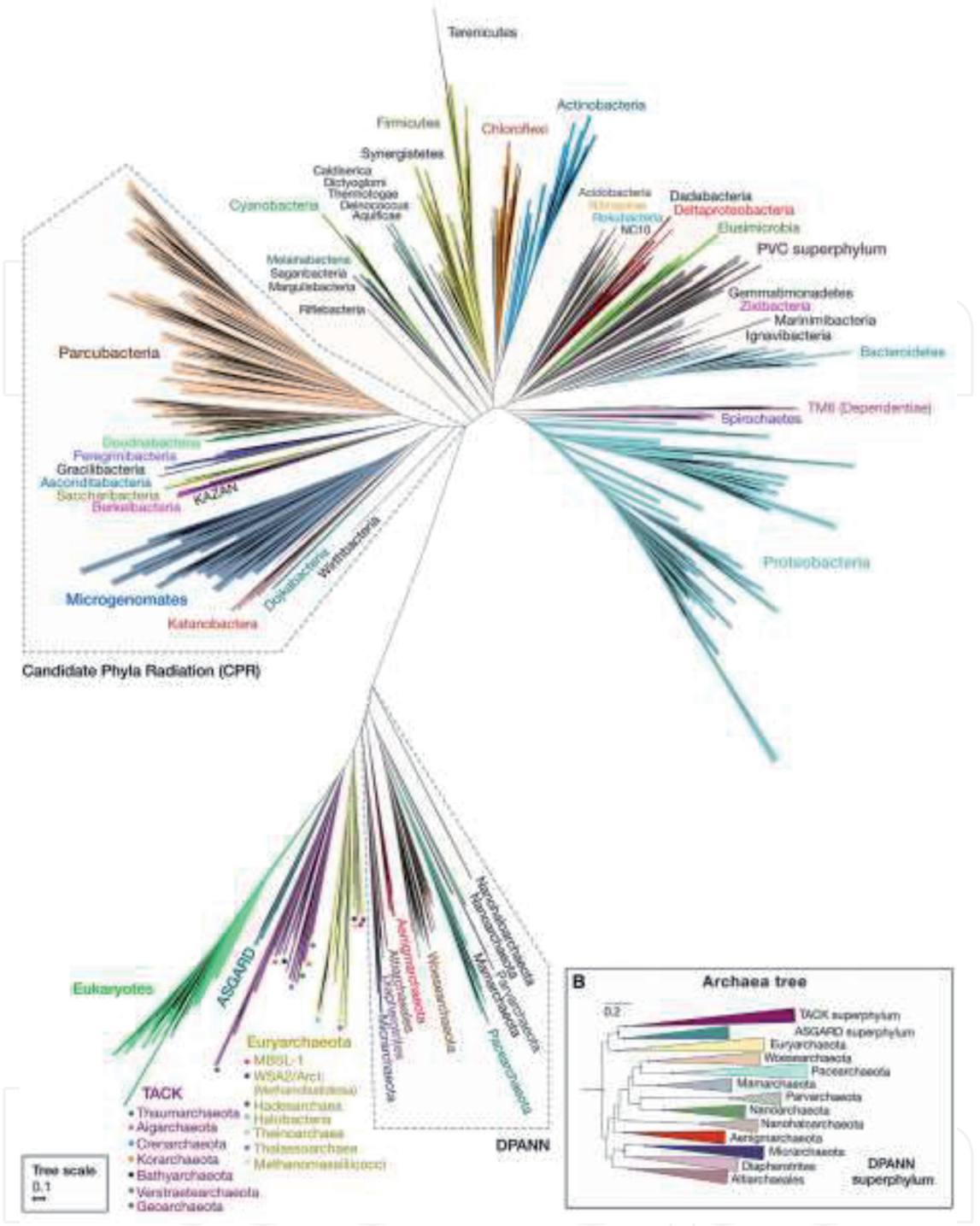

Figure 1.
Representation of the tree of life based on SSU rDNA gene sequences of the three domain of life [23].

3. Antimicrobial potential of extremely halophilic archaea

Halophilic archaea were the first members of archaea found to produce bacteriocins-like proteins known as halocins. The first studies date from the beginning of 1980s with experiments demonstrating the presence of antagonistic interactions between halophilic archaeal strains isolated from the Alicante salt in Spain [20]. Today, about fifteen halocins have been described and only three genes, *halC8*, *halS8* and *halH4*, coding C8, S8 and H4 halocins, respectively, have been identified [21, 22]. Yet, no three-dimensional structural data of archaeocins are available in public databases.

3.1 Halocins

Halocins, bacteriocins-like peptides and proteins produced by extremely halophilic archaea, were first discovered in 1982 by F. Rodriguez Valera [24, 25]. They are classified according to their size into two major classes: high molecular mass (protein, > 10 kDa) and low molecular mass (peptide, ≤10 kDa) called microhalocins [26, 27]. It has been shown that halocins are effective against *Haloarchaea* and *Crenarchaea* such as *Sulfolobus* spp. and *Methanosarcina thermopila*, and thus act across the main subdivision of the archaeal domain. These compounds represent a general class of antiarchaeal toxins and there is no confirmation about the inhibition of bacteria [26, 27]. Production of halocin is a practically universal feature of archaeal halophilic rods [27]. Although several halocins were identified, only some of them have been characterized and purified.

3.1.1 Microhalocins

These halocins are composed of a peptide with size below or in the range of 10 kDa. Seven halocins have been characterized including HalS8, HalR1, HalC8, HalU1, HalH6, Sech7a and Sech10. They are hydrophobic and retain their activity in the absence of salt and can be stored at 4°C. They are relatively insensitive to heat and organic solvents [28].

3.1.1.1 Halocin S8 (HalS8)

HalS8 is the first characterized microhalocin with 36 amino acids (3580 Da), it is synthesized by the uncharacterized S8a haloarchaea [29]. Halocin S8 showed a narrow inhibitory spectrum and can only inhibit *Halobacterium salinarum* NRC817, *Halobacterium* GRB and *Haloferax gibbonsii* [29]. It can be desalted and it is heat resistant. Its activity is resistant to trypsin but sensitive to proteinase K and is undetectable in the transition to the stationary phase [29, 30]. The *halS8 gene* is *encoded* on a ~200-kbp megaplasmid [29].

3.1.1.2 Halocin HalR1 (HalR1)

Halocin R1, the second characterized microhalocin, is produced by *Halobacterium salinarum* GN101, a strain isolated from solar salt marsh in Mexico [31]. Initially HalR1 was described with a molecular weight of 6.2 kDa [32] and later on, it was shown that the HalR1 peptide is composed of 38 amino acids [24, 29]. Like HalS8, the activity is not affected by desalting and is resistant to acids, bases, organic solvents DNase and RNase, and against some proteases such as papain, trypsin or thermolysin, but it is sensitive to proteinase K, pronase P and elastase [20, 32].

3.1.1.3 Halocin C8 (HalC8)

Halocin C8 is produced by *Natrinema* sp. AS7092, a strain isolated from the large Chaidan Salt Lake, China (7.44 kDa, 76 amino acids) [28]. It is a unique polypeptide with an isoelectric point of 4.4 [33]. Its activity is retained after desalting, boiling and frozing [33]. Halocin C8 has a very broad spectrum of activity against several species and genera of *Halobacteriales* members including *Natronobacterium gregoryi*, *Nbt*. comb. nov and *Natronomonas pharaonis* [28]. The *halC8* gene encodes both halocin C8 and its immunity protein HalI.

3.1.1.4 Halocin A4 (HalU1)

Halocin A4, also called also halocin U1, is produced by an uncharacterized haloarchaea strain isolated from a Tunisian saltern [34]. Its molecular weight is 7.435 Da, as determined by the spectrometric mass, and is both acidic (pH = 4.14) and hydrophobic (eluent at ~85% acetonitrile) [26]. Halocin A4 has been reported to inhibit the growth of crenarchaeal *Sulfolobus* sp. strains [26]. G*ene* encoding HalA4 is *located* on a 300 kpb megaplasmid, pHM300 (NC_017943) [29].

3.1.1.5 Halocin H6 (HalH6)

Halocin H6 is produced by *Haloferax gibbonsii* Ma 2.39 species [27]. Its activity is resistant to trypsin. Stabilities of this peptide were studied and have shown that HalH6 can be desalted and it retained its activity after heat treatment up to 10 min at 100°C [27]. Halocin H6 is considered as a bactericidal substance which causes cell lysis and the specific target of HalH6 is the Na+/H+ antiport [27, 35].

3.1.1.6 Halocin Sech7a

Halocin Sech7a was excreted by the extremely halophilic haloarchaeon Sech7a, isolated from brine samples of Secovlje solar salterns crystallizers in Slovenia [36]. Sech7a is about 11 kDa. It is stable over a wide pH range and is heat labile at temperatures above 80°C. Its optimal activity was observed in the early exponential phase growth at 45°C. It loses activity under low salt conditions, but its activity can be restored after dialysis against initial saline conditions [36].

3.1.1.7 Halocin SH10

Halocin SH10 is produced by *Natrinema* sp. BTSH10, a strain isolated from the Kanyakumari salt marsh, Tamil Nadu, India [37]. The optimal production of halocin SH10 is at 42°C, pH 8.0 and 3 M NaCl at the stationary phase. In this context, it was reported that the activity is lost under acidic conditions [37]. Production of SH10 is influenced by the carbon source composition of the medium — *Natrinema* sp. BTSH10 could produce maximal halocin in the presence of beef [37].

3.1.2 Protein halocins

This class comprises halocins composed of proteins greater than 10 kDa in size. Currently, there are two characterized protein halocins, HalH1 and HalH4, in the range of 30 to 35 kDa [28].

3.1.2.1 Halocin H4

Halocin H4, produced by *Haloferax mediterranei* R4 (ATCC 33500) was isolated from a Spanish solar salt pond in Alicante. It is the first halocin that was studied [20]. The optimal activity was detected at the midpoint between exponential and stationary phases [20]. Halocin H4 is sensitive to proteases, high temperature and desalting. HalH4 has an antimicrobial activity against other haloarchaeons. It Interacts with the membrane of the target cells where it causes permeability changes that result in an ionic imbalance leading to cell lysis and death [21, 38]. The *halH4* gene, encoding halocin activity, is located on the pHM300 megaplasmid, a single polypeptide of 34.9 kDa.

3.1.2.2 Halocin H1

Halocin H1 is produced by *Haloferax mediterranei* M2a (previously known as *H. mediterranei* Xia3) isolated from salt ponds in Santa Pola (Alicante, Spain) [20]. Halocin H1 is a single 31 kDa polypeptide characterized by a broad inhibitory spectrum among *Halobacteriales* members. HalH1 activity is temperature and salt dependant. It is stable at 50°C only and requires a salt concentration of 1.5 M to maintain its activity [38, 39]. Optimum activity was observed at mid-exponential phase. The sensitivity to proteases and the gene encoding activity were not determined yet.

3.2 Applications of halocins

Some studies reported the role of halocins in a variety of environmental, industrial and biotechnological applications ***(REFERENCES?). However, this topic is poorly documented and somewhat controversial. One of these applications is the use of halocin producing strains in the textile industry during the tanning process characterized by high salinity concentration, halocins could inhibit the growth of pathogenic microbes affecting the quality of products. [7, 10]. Moreover, some halocins have also been reported for biomedical and therapeutic uses, for example, Halocin H7 has been shown to inhibit the Na+/H+ antiport in *Haloarchaea*, can be used as a treatment to reduce the injuries caused when ischemic organ transplantation is re-infused [35]. The therapeutic potential of halocins needs more research on their physical structures and their modes of action. On the other hand, halocins are known also to have a potential application in food industry as preservative agents by controlling the growth of haloarchaea in salted food products [40].

4. Materials and methods

Here, we evaluated the evolutionary relationship between bacteriocin- like-producing haloarchaea members based on comparisons of their amino acid sequences retrieved from annotated genomes sequences deposited in the IMG database [41].

4.1 Database search of halocin gene clusters

Schematic workflow of the methodology employed of amino acid sequences retrieving and phylogenetic assessment is illustrated in **Figure 2**. The methodology consisted of: first, complete and nearly complete genome sequences of *Halobacteriales* members were retrieved from IMG database. Then, in *silico* screening for gene sequences encoding halocins was done based on the BLASTP algorithm with default parameters [42]. All redundant and low-quality sequences were eliminated from datasets.

4.2 Phylogenetic reconstruction

Multiple sequences alignment of retrieved amino acid sequences were performed using ClustalW [43]. The evolutionary history was inferred using the Unweighted pair group method with arithmetic mean (UPGMA) method [44] implemented in MEGA X [45, 46]. The optimal tree with the sum of branch length = 18.99 is shown. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [47] and are in the

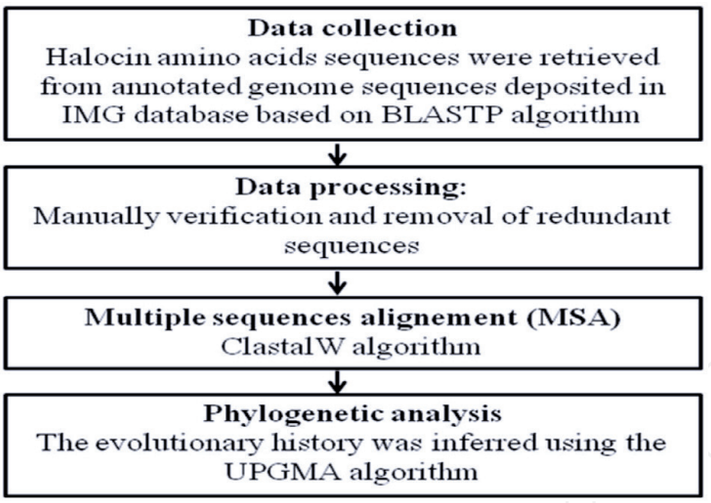

Figure 2.
Schematic workflow of the methodology employed for amino acids sequences retrieving and phylogenetic assessment.

units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). In the final dataset, a total of 405 positions was obtained.

5. Results

5.1 Amino acid sequence of halocins

A total of 1858 of complete and nearly complete genome sequences of *Halobacteriales class* members were retrieved from the IMG database and creened for halocin encoding gene based on the BLASTP algorithm with default parameters [42].

A total of 61 amino acid sequences were retrieved from 15 genera belonging to *Halobaceria* class including *Natrinema*, *Haloferax*, *Haloterrigena*, *Natronorubrum*, *Halobacterium*, *Haladaptatus, Halorubrum, Halococcus, Halopiger*, *Natrialba, Halolamina*, *Natronococcus, Haloarcula, Halapricum and Halorussus*. Furthermore, some other unclassified halohilic archaea were present as well, including uncultured halophilic archaeon, halophilic archaeon sp. DL31 and Haloarchaeon S8 (**Table 1**).

Results showed that some species present more than one copy for halocin encoding genes. In fact, three (n = 3) classes of halocins were identified in this study (**Table 1**).

The first class is halocin C8-like bacteriocin domain (HalC8), the best known bacteriocin like sequences in archaea, it has been demonstrated to be produced from a ProC8 precursor, targeted to the membrane by the Tat pathway, and cleaved by an unknown mechanism to yield the active mature peptide HalC8 and an immunity protein HalI, protecting the producing strain against its own AMP [22]. HalC8 was identified in all species except *Natrialba aegyptia* DSM 13077. Indeed, *Natrinema* genus members appears to be more represented in terms of C8-like bacteriocin production. It's worth noting that among the six officially described species within the *Natrinema* genus, five are described in the current analysis. Several studies report the production of HalC8 and/or the presence of the *halC8* gene among *Natrinema* species isolated from different geographical origins like Chaidan Salt Lake in Qinghai province, China [33], Ichekaben salterns Chotts and sebkhas in algeria [48, 49].

The second class is halocin H4 (HalH4) identified in *Haloferax mediterranei* strain ATCC 33500, *Natrialba aegyptia and Natrinema gari* JCM 14663 species.

Taxonomy	Genus	Species level	Class of Halocins
Domain: *Archaea* Kingdom: *Euryarchaeota* Phylum: *Euryarchaeota* Class: *Halobacteria* Order: *Halobacteriales* Family: *Halobacteriaceae*	*Natrinema*	*Natrinema pellirubrum* 157 JCM 10476	Halocin C8-like bacteriocin
		Natrinema sp. J7-2	
		Natrinema pallidum DSM 3751	
		Natrinema pellirubrum DSM 15624	
		Natrinema altunense 1A4-DGR	
		Natrinema altunense JCM 12890	
		Natrinema sp. J7-1	
		Natrinema altunense AJ2	
		Natrinema atunense 4.1	
		Natrinema gari JCM 14663	Halocin C8-like bacteriocin and Halocin H4
	Haloferax	*Haloferax mediterranei* ATCC 33500	Halocin H4
		Haloferax mediterranei R-4	
		Haloferax lucentense DSM 14919	Halocin C8-like bacteriocin
		Haloferax sp. ATCC BAA-646	
		Haloferax volcanii DS2	
		Haloferax alexandrinus JCM 10717	
		Haloferax sp. ATCC BAA-645	
		Haloferax larsenii CDM 5	
		Haloferax larsenii JCM 13917	
	Haloterrigena	*Haloterrigena thermotolerans*	Halocin H4
		Haloterrigena salifodinae ZY19	Halocin C8-like bacteriocin
		Haloterrigena jeotgali A29	
		Haloterrigena mahii H13	
		Haloterrigena salina JCM 13891	
		Haloterrigena sp. P1A	
		Haloterrigena turkmenica WANU15	
	Natronorubrum	*Natronorubrum tibetense* DSM 13204	Halocin C8-like bacteriocin
		Natronorubrum tibetense GA33	
		Natronorubrum sediminis CGMCC 1.8981	

Taxonomy	Genus	Species level	Class of Halocins
Domain: *Archaea* Kingdom: *Euryarchaeota* Phylum: *Euryarchaeota* Class: *Halobacteria* Order: *Halobacteriales* Family: *Halobacteriaceae*	*Halobacterium*	*Halobacterium* sp. DL1	Halocin C8-like bacteriocin
		Halobacterium salinarum DSM 670	
		Halobacterium salinarum DSM 671	
		Halobacterium salinarum DSM 6692	
		Halobacterium salinarum DSM 3754	
		Halobacterium salinarum DSM 668	
	Haladaptatus	*Haladaptatus paucihalophilus* DX253	
		Haladaptatus sp. R4	
		Haladaptatus paucihalophilus DSM 18195	
	Halorubrum	*Halorubrum lacusprofundi* R1S1	
		Halorubrum trapanicum CBA1232	
	Halococcus	*Halococcus* sp. 197A	
		Halococcus salifodinae DSM 8989	
	Halopiger	*Halopiger* sp. IIH3	
	Natrialba	*Natrialba aegyptia* DSM 13077	
	Halolamina	*Halolamina pelagica* CGMCC	
	Natronococcus	*Natronococcus occultus* SP4	
	Haloarcula	*Haloarcula salaria* H5-DGR	
	Halapricum	*Halapricum salinum* CBA1105	
	Halorussus	*Halorussus amylolyticus YC93*	
	Halophilic archaeon	halophilic archaeon sp. DL31	
	uncultured halophilic archaeon	uncultured halophilic archaon J07HX5	
		uncultured haloarchaeon J07ABHX67	
		Uncultured Halobacteriaceae archaea SG1_71_5	
	Haloarchaeon S8a	*Haloarchaeon S8a*	Halocin S8

Table 1.
Classes of Halocins identified by in silico analysis of all genomes of halophilic archaea domain available in IMG database.

HalH4 was first characterized from *H. mediterranei* isolated from solar saltern lakes of Spain [50]. HalH4 is a 40 kDa protein with an N-terminal 46 aa leader peptide which is cleaved off leaving a 313 aa mature halocin [51].

The third class is halocin S8, a microhalocin of 36 amino acids (3580 Da) initially purified from an unidentified haloarchaeal strain S8a, isolated from the Great Salt Lake (Utah, 109 United States) [52].

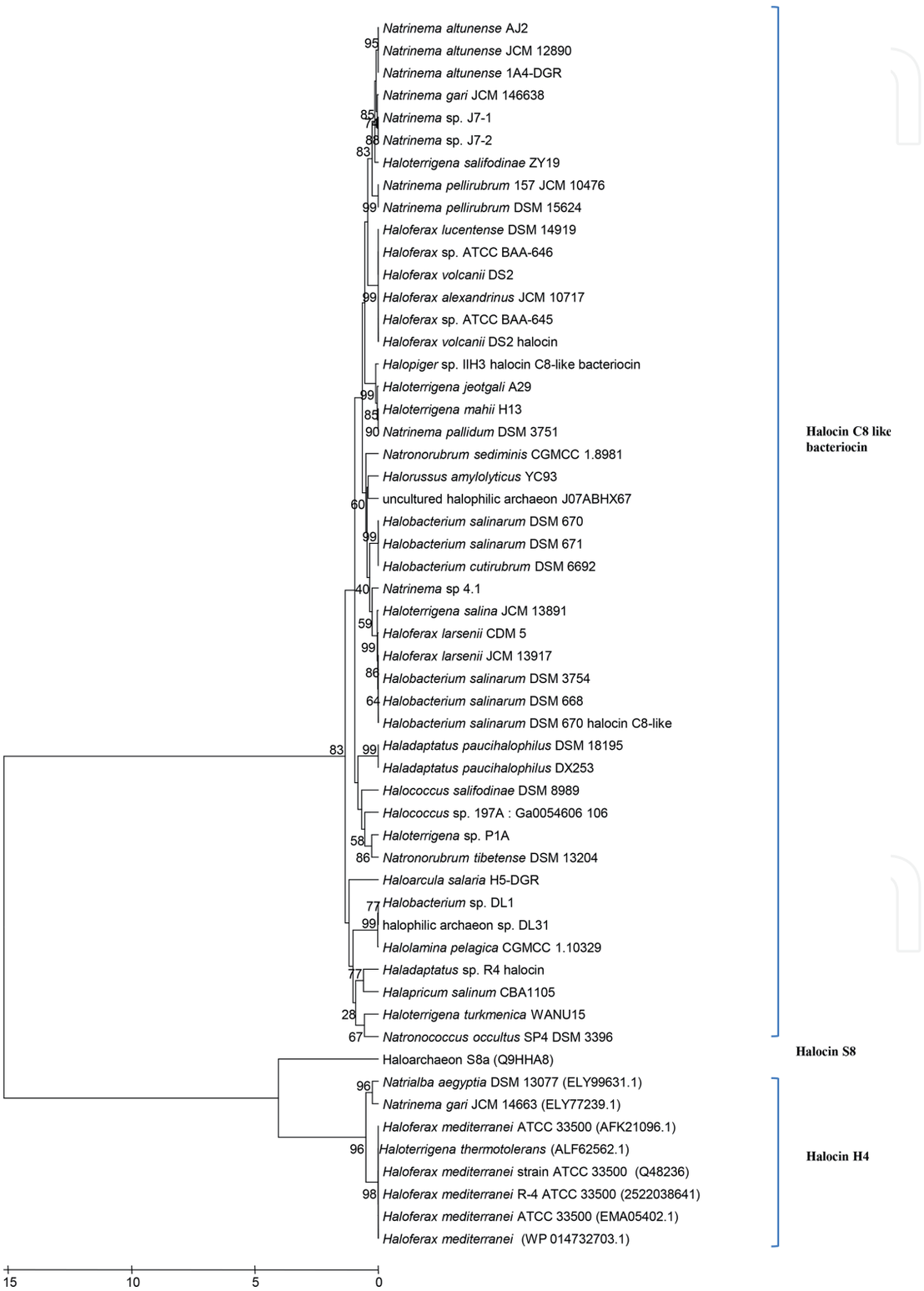

Figure 3.
Phylogenetic tree of halocin amino acid sequences of halophilic archaeal species. The evolutionary history was inferred using the UPGMA algorithm implemented in MEGA X software. Numbers at the nodes indicate the percentage of occurrence in 100 bootstrapped trees (bootstrap values > 50% are shown).

5.2 Phylogenetic analysis

Phylogenetic analysis of retrieved halocin peptide sequences was conducted and the result is illustrated in **Figure 3**. Results showed a clear segregation of the three halocins classes (C8, H4 and S8), where halocin S8 is phylogenetically more close to HalH4. Furthemore, no clear separation of species was observed based on HalC8 amino acids sequences analyses. HalC8 was detected in 12 genera belonging to three orders of *Halobacteria* class [53]: *Natrialbales* (*Natrinema*, *Haloterrigena*, *Natronorubrum*, *Halopiger*); *Haloferacales* (*Haloferax*, *Halolamina*) and *Halobacteriales* (*Halorussus*, *Halobacterium*, *Haladaptatus*, *Halococcus*, *Haloarcula*, *Halapricum*) and uncultured halophilic archaeon (J07ABHX67) phylogenetically related to species *Halorussus amylolyticus* YC93. The halocin S8 was detected only in the strain Haloarchaeon S8a (Q9HHA8). HalH4 is identified in *Natrialbales* (*Natrinema gari* JCM 14663 (ELY77239.1), *Haloterrigena thermotolerans*, *Natrialba aegyptia* DSM 13077) and *Haloferacales* (*Haloferax*).

It's worth noting that HalH4/HalC8 halocins were identified in *Haloferax, Haloterrigena* and *Natrinema* genera with only the species *Natrinema gari* JCM 14663 (ELY77239.1) being able to produce the two classes in the same time. Earlier studies reported that several described halocins, with broad inhibitory properties, are derived from *Haloferax* and *Natrinema* strains [24, 28, 45, 51, 54] and it has been suggested that halocin production may explain their dominance in some saline ecosystems [54, 55].

6. Conclusion

On the basis of our *in silico* analyses, we can conclude that halocin production is considered as a general feature of some members of halophilic archaea, particularly members of *Natrialbales* and *Haloferacales* orders with the occurrence of Halocin C8-like production. This group can thrive in saline ecosystems in which several other microorganisms are not able to live. Thus, the dominance of certain species isolated in some saline ecosystems could be attributed to halocin production as a mechanism of competition between microrganisms. This chapter will open new research lines that will shed light on halocins as marker for haloarchaeal phylogentic delineation.

Conflict of interest

We have *no conflict of interest* to declare.

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