# 5 Peptide and Protein Antibiotics from the Domain *Archaea*: Halocins and Sulfolobicins

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## Summary

Production of peptide or protein antibiotics is a near-universal feature in all three domains of life. While bacteriocins and eucaryocins have been studied for decades, research in the field of archaeocins (halocins and sulfolobicins) is just emerging; most *Archaea* have yet to be screened for antibiotic production. To date, only seven halocins and one sulfolobicin have been partially or fully characterized, but antagonism studies suggest that there are hundreds of different halocins. Halocins are diverse in size (ranging from 3–35 kDa), thermal stability, and salt-dependence. Their activity spectra are typically "broad" with respect to killing other haloarchaea, and some microhalocins (small peptide halocins) have demonstrated cross-phylum inhibition. Currently, the mechanism of action is known only for halocin H6/H7, which inhibits the $Na^+/H^+$ antiporter in both haloarchaeal and mammalian cells. The potential biotechnological applications of other halocins will hinge on discovery of their mechanisms of action.

## 5.1 Introduction

In contrast to the wealth of studies for bacteriocins that began in 1925 (Gratia 1925) and have been chronicled in this volume, the characterization of peptide and protein antibiotics from organisms that inhabit the domain *Archaea* ("archaeocins") is only beginning (O'Connor and Shand 2002) – the first report of an archaeocin was published in 1982 (Rodriguez-Valera et al. 1982). The term "archaeocin" was coined to distinguish peptide and protein antibiotics produced by *Archaea* from those produced by members of the domain *Bacteria* (Price and Shand 2000). To refer to archaeocins as bacteriocins

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perpetuates the confusion between these two domains of prokaryotic organisms: *Archaea* are no more closely related to *Bacteria* than are *Eucarya* (Woese et al. 1990). Having made this distinction, it is logical to include a chapter on archaeocins in a text devoted to bacteriocins, as the archaeocin field is just emerging and grouping the prokaryotic antimicrobial producers together makes sense. In addition, the term "halobacteria" was used early on as a collective term that encompassed all extremely halophilic members of the domain *Archaea* (i.e., members of the archaeal family *Halobacteriaceae*) and not, as one would assume, as a reference to halophilic members of the domain *Bacteria*. Subsequently, this terminology has been replaced by the term "haloarchaea", preventing further confusion. Continuing with the same nomenclature, peptide and protein antibiotics produced by members of the domain *Eucarya* are called "eucaryocins" (O'Connor and Shand 2002), with the first reports appearing in the early 1960s. Consequently, there is a plethora of information about these protein antibiotics as well (see http://www.bbcm.univ.trieste.it/ for an up-to-date list of 880+ eucaryocins).

To date, archaeocins have been characterized from only two phylogenetic groups: euryarchaeal extreme halophiles (haloarchaea) that produce "halocins" (O'Connor and Shand 2002), and the crenarchaeal genus *Sulfolobus*, an aerobic hyperthermophile that produces a "sulfolobicin" (Prangishvili et al. 2000). Although "production of halocin is a practically universal feature of archaeal halophilic rods" (Torreblanca et al. 1994), and based upon antagonism studies (Meseguer et al. 1986; Torreblanca et al. 1994), there appear to be hundreds of different halocins, only a handful of these have been characterized (see Table 5.1). Halocin protein sequences are unique, as they do not match anything in the protein sequence databases. Unfortunately, of the four haloarchaeal genomes that have been sequenced (*Halobacterium* sp. NRC-1: Ng et al. 2000; *Haloferax volcanii*: www.tigr.org/tdb; *Haloarcula marismortui*: Baliga et al. 2004; and *Natronomonas pharaonis*, an alkaliphilic haloarchaeon: Falb et al. 2005), none is a halocin producer (see Sect. 5.2.1). At the moment, halocin research must take place in the absence of a fully sequenced genome containing a halocin gene. Despite this limitation, all haloarchaea are aerobes and are easy to grow, with typical generation times between 1.5 and 3 h (Robinson et al. 2005). Detailed protocols for isolating microhalocins are also available (Shand 2006), as is a complete bibliography of the halocin literature (http://jan.ucc.nau.edu/~shand). What this field needs now are more scientists.

## 5.2 Halocins

### 5.2.1 The Ubiquity of Halocin Production

In 1992, J.R. Tagg posited that bacteriocin production would be a near-universal feature of bacteria, given a sufficient number of indicator strains

**Table 5.1** Halocin characteristics (this table is reprinted with permission from Springer; O'Connor and Shand 2002)

| Halocin | Producer (source) | Size | GenBank accession # | Thermal stability | Salt dependent | Activity spectrum[a] | Mechanism | References |
|---|---|---|---|---|---|---|---|---|
| A4 | Haloarchaeon TuA4 (solar saltern, Tunisia) | 7,435 Da | - | ≥ 1 week at boiling[b] | No | Broad *Sulfolobus* spp. | ND | Haseltine et al. (2001), Duncan (2004) |
| C8 | *Halobacterium* strain AS7092 (Great Chaidan Salt Lake, China) | ~31.1 kDa (prepro-protein; ProC8), 7,427 Da (mature; HalC8) | AY310321 | > 60 min at 100 °C | No | Broad | ND | Li et al. (2003), Sun et al. (2005) |
| G1 | *Halobacterium* strain GRB (solar saltern, France) | ND[c] | - | ND | ND | Broad | ND | Soppa and Oesterhelt (1989) |
| H1 | *Haloferax mediterranei* M2a (previously Xia3; solar saltern, Spain) | 31 kDa | - | < 50 °C | Yes | Broad | Membrane permeability | Rodriguez-Valera et al. (1982), Meseguer et al. (1986), Platas (1995), Platas et al. (1996), Platas et al. (2002) |
| H2 | Haloarchaeon Gla2.2[d] (solar saltern, Spain) | ND | - | ND | ND | Broad | ND | Rodriguez-Valera et al. (1982) |
| H3 | Haloarchaeon Gaa 12 (solar saltern, Spain) | ND | - | ND | ND | Broad | ND | Rodriguez-Valera et al. (1982), Meseguer et al. (1986) |

(*Continued*)

**Table 5.1** Halocin characteristics (this table is reprinted with permission from Springer; O'Connor and Shand 2002)—Continued

| Halocin | Producer (source) | Size | GenBank accession # | Thermal stability | Salt dependent | Activity spectrum[a] | Mechanism | References |
|---|---|---|---|---|---|---|---|---|
| H4 | *Haloferax mediterranei* R4 (solar saltern, Spain) | 39.6 kDa (preprotein), 34.9 kDa (mature) | U16389 | < 60 °C | Partially[f] | Broad | Proton flux? | Rodriguez-Valera et al. (1982), Meseguer and Rodriguez-Valera (1985), Meseguer and Rodriguez-Valera (1986), Meseguer et al. (1986), Meseguer et al. (1995), Cheung et al. (1997), Shand et al. (1999), Perez (2000) |
| H5 | Haloarchaeon Ma 2.20[d] (solar saltern, Spain) | ND | - | ND | ND | Narrow | ND | Rodriguez-Valera et al. (1982) |

(*Continued*)

**Table 5.1** Halocin characteristics (this table is reprinted with permission from Springer; O'Connor and Shand 2002)—Continued

| Halocin | Producer (source) | Size | GenBank accession # | Thermal stability | Salt dependent | Activity spectrum[a] | Mechanism | References |
|---|---|---|---|---|---|---|---|---|
| H6/H7 | *Haloferax gibbonsii* Ma2.39[e] (solar saltern, Spain) | ~3 kDa | - | ≤ 90 °C | No | Broad | $Na^+/H^+$ antiporter inhibitor | Rodriguez-Valera et al. (1982), Meseguer et al. (1986), Torreblanca et al. (1989), Meseguer et al. (1995), Alberola et al. (1998) |
| R1 | *Halobacterium* strain GN101 (solar saltern, Mexico) | 3.8 kDa | - | 60 °C | No | Broad *Sulfolobus* spp., *M. thermophila* | ND | Rdest and Sturm (1987), Shand et al. (1999), Haseltine et al. (2001), O'Connor (2002) |
| S8 | Haloarchaeon S8a (Great Salt Lake, UT) | 33.9 kDa (prepro-protein), 3.58 kDa (mature) | AF276080 | ≥ 24 h at boiling[b] | No | Broad *Sulfolobus* spp. | ND | Shand et al. (1999), Price and Shand (2000), Haseltine et al. (2001) |

[a]Activity spectrum refers to inhibition of haloarchaea, unless otherwise indicated. For a definition of "broad", see Sect. 5.2.3
[b]This study was done at 2,113 m (7,000 ft); water boils at 93 °C at this elevation
[c]ND: not determined
[d]Isolates Gla2.2 and Ma2.20 were labeled as GLA22 and MA220, respectively, in Rodriguez-Valera et al. (1982)
[e]Halocin H6 is produced by *Haloferax gibbonsii* Ma2.39 and was first reported as a 32-kDa protein, but is now known to be a microhalocin of about 3 kDa. *Hfx. gibbonsii* Ma2.39 is proprietary and should not be confused with a different halocin-producing strain, *Hfx. gibbonsii* Ma2.38 (ATCC 33595). Halocin H7 is halocin H6, but is produced by a halocin-overproducing mutant of *Hfx. gibbonsii* Ma2.39 called *Hfx. gibbonsii* Alicante SPH7
[f]See Sect. 5.2.4

(Tagg 1992). Similarly, the diversity of eucaryal organisms that produce antimicrobial peptides is vast, ranging from protozoans to plants to humans (O'Connor and Shand 2002). After conducting two non-overlapping antagonism studies (Meseguer et al. 1986: 79 isolates; Torreblanca et al. 1994: 68 isolates), Torreblanca et al. reached the same conclusion regarding halocins: "Production of halocin is a practically universal feature of archaeal halophilic rods" (Torreblanca et al. 1994). In all, of the 147 isolates screened, only three failed to show any inhibitory activity.

However, there are three issues that surround these two antagonism studies. First, only a single medium with one salt concentration (25% (w/v) marine salts) was used to grow all of the isolates; no attempt was made to use optimal NaCl concentration(s) for growth of any of the isolates. As mentioned in the studies, this resulted in wide variations in growth rates. Second, it is not clear that all activities were due to peptides or proteins. In the 1986 study, the 79 isolates were assigned to one of 15 groups based in part on their activity spectrum. However, only supernatants from "representatives" of these 15 groups were subjected to protease inactivation. The 1994 study does not state unequivocally that all cells or culture supernatants demonstrating inhibitory activities were treated with proteases, but they very well may have been. Third, the 1986 study indicated that *Haloferax volcanii* DS2 inhibited six of the 79 isolates, three of which were culture collection strains of the same genus: *Har. vallismortis*, *Har. marismortui* (previously *Hbt. marismortui*) and *Har. hispanica* (previously *Hbt. hispanicum*). However, in the Shand laboratory, *Hfx. volcanii* DS2 does not inhibit any of these three strains. In addition, scrutiny of the *Har. marismortui* genome and the *Nmn. pharaonis* genome does not reveal any obvious halocin sequences. Moreover, these two strains inhibited only a single member of the 79-member collection in the antagonism study (Meseguer et al. 1986). These differences may be due simply to differences between the various isolates in the strain collections.

### 5.2.2 The Role of Halocins in the Environment and the Inability to Detect Halocin Activity in Hypersaline Crystallizer Ponds

Given the ubiquity of halocin production described above, one might predict that aquatic hypersaline environments might be replete with halocin activity. To test this hypothesis, Kis-Papo and Oren (2000) sampled four different crystallizer ponds; two ponds were sampled only once whereas the other two were sampled repeatedly and at different times of the year. These ponds contained large numbers of prokaryotic microorganisms ($8.4 \times 10^6 - 7.2 \times 10^8$, by direct cell counts) dominated by haloarchaea. Using 12 haloarchaeal indicator strains representing five genera, cell-free brines showed no evidence of halocin activity, regardless of the pond, even when some of the brines were concentrated as much as 53.5-fold. From one pond in Eilat, Israel, a collection of 41 haloarchaea were isolated, 29 of which showed halocin activity against at least one of the 12 indicator strains, demonstrating that halocin producers were present in

the pond. The authors concluded that "One possibility is that under field conditions no significant quantities of halocins are produced and that halocins are unimportant in interspecies competition in hypersaline lakes" (Kis-Papo and Oren 2000). They added that halocins might have been present in the brines, but they might have bound non-specifically to the filter membranes (although control experiments suggest that this was not an issue) or they may have been degraded by proteases present in the brines during transport.

Another possibility is that halocins are produced in crystallizer ponds, but as soon as they bind to a target (or even bind non-specifically to debris?), they become inactive. To determine how quickly halocin activity would disappear, preliminary "disappearing halocin activity" experiments involving mixing halocin-laden supernatants with halocin-sensitive cells in broth have been performed (O'Connor and Shand 2002). Samples were removed periodically and assayed for halocin activity. The activity of halocin A4 disappeared in less than a minute, halocin R1 activity was reduced in as little as 5 min with some activity remaining after 24 h, and halocin S8 activity did not diminish at all. It is unknown if these preliminary experiments were conducted under saturating concentrations of halocin-to-target.

The teleological explanation for prokaryotic antimicrobial production in the environment has been to reduce competition and/or lyse cells to acquire nutrients. For halocins, evidence supporting the latter part of this model has been found by Platas et al. (1996). A halocin producer (*Hfx. mediterranei* Ma2, formerly Xia3) was mixed with a non-producer (*Hbt. salinarum*) in the absence of any nutrients. The producer strain was able to grow, presumably through the release of cellular contents of the non-producer.

However, the concept that antimicrobial production reduces competition (and therefore diversity) is being challenged; the presence of antimicrobials in the environment is thought to maintain or even increase species diversity through a rock-paper-scissors model (Lenski and Riley 2002; Kirkup and Riley 2004; see Chap. 6, this volume). In this model, which organism(s) dominates may change over time, and although some organisms may become rare, they nevertheless persist and do not disappear. For example, in the early phylogenetic placement of the haloarchaea, three organisms isolated from a solar saltern in Alicante, Spain (*Hfx. mediterranei*, *Hfx. gibbonsii* and *Har. hispanica*) helped to define three of the founding genera in the family *Halobacteriaceae*. Upon returning to the site a couple of years later, none of these organisms was recovered by culturing or by PCR (Rodríguez-Valera et al. 1999). Are these organisms gone, or have they simply become rare? If they have become rare, why is that? Furthermore, the presence of antimicrobials may generate sufficient selective pressure for spontaneous antimicrobial-resistant mutants to arise.

### 5.2.3 Activity Spectra

Activity spectra (or killing breadth) tend to be relatively narrow in bacteriocins, being limited to bacteria closely related to the producing strains (Riley

and Wertz 2002). However, nisin, a lactococcal lantibiotic, inhibits the crenarchaeal hyperthermophile *Sulfolobus acidocaldarius* (P.D. Clark and D.W. Grogan, personal communication). This is the first example of *Bacteria*/*Archaea* cross-domain inhibition.

Table 5.1 includes the activity spectra of halocins that have been characterized either fully or partially. Initial reports describing a particular halocin frequently use a relatively small number of characterized haloarchaeal strains from culture collections to determine its activity spectrum (e.g., Rodriguez-Valera et al. 1982; Kis-Papo and Oren 2000; Li et al. 2003). Halocins H1, H2, H3, H4, H5 and H6/H7 have been retested against 79 haloarchaeal strains in the 1986 antagonism study. All six have a "broad" activity spectrum when it comes to inhibiting haloarchaeal isolates (inhibiting between 63–74; Meseguer et al. 1986). Together, activity spectra in the two antagonism studies ranged from strains that inhibited zero to those that inhibited as many as 74 strains.

In order to challenge gram-positive and gram-negative bacteria, halocins have to be desalted without losing activity, limiting the population of testable halocins to the microhalocins (see Sect. 5.2.4). No microhalocin has been shown to inhibit any bacterial organism. This is not an unexpected result, as the microhalocins that have been characterized have either little or no net charge and are unable to interact with the negatively charged bacterial membrane the way that many bacteriocins and eucaryocins do. Similarly, microhalocins do not inhibit lower eukaryotic microorganisms, including *Saccharomyces* spp. However, halocin H6/H7, which inhibits the haloarchaeal $Na^+/H^+$ antiporter (Meseguer et al. 1995), also inhibits the $Na^+/H^+$ antiporter in a dog model (Alberola et al. 1998). It is not known if halocin H6/H7 inhibits $Na^+/H^+$ antiporters in organisms evolutionarily intermediate between haloarchaea and mammals.

Halocins A4, R1 and S8 all inhibit *Sulfolobus* spp. (crenarchaeal hyperthermophiles that grow optimally at 80 °C and pH 3), with halocin R1 also inhibiting *Methanosarcina thermophila* (a mesophilic methane-producing euryarchaeote; Haseltine et al. 2001; Table 5.1). Indeed, this is a broad spectrum of activity representing cross-phylum inhibition, as haloarchaea are in the phylum Euryarchaeota whereas *Sulfolobus* spp. are in the phylum Crenarchaeota. It may be that other halocins can inhibit distantly related archaeal organisms, but they have yet to be tested for this breadth of inhibition.

From a hypersaline field site in Utah, we have isolated more than 350 different extreme halophiles spanning all three domains, as determined by amplification of 16S or 18S rDNA sequences using domain-specific primers (P.J. Polsgrove, B.A. Roberts, M.A. Mishler, R.F. Shand, unpublished data). Preliminary antagonism studies employing 48 purified isolates show that 62% inhibited at least one of the other isolates, with some isolates inhibiting as many as 30 of the other 47 strains. This is consistent with the Kis-Papo and Oren study (2000) where 71% (29/41) of the isolates from the crystallizer pond in Eilat inhibited at least one member of the 12 tester strains. Despite

the large number of antimicrobial producers at the Utah site, the microbial diversity appears very high, supporting the argument that antimicrobial production may contribute to the maintenance or enhancement of species diversity. Polsgrove et al. (P.J. Polsgrove, B.A. Roberts, M.A. Mishler, R.F. Shand, unpublished data) also found bacterial extreme halophiles are inhibiting haloarchaea, haloarchaea are inhibiting bacterial extreme halophiles, and there are several extremely halophilic fungi that inhibit both bacterial and archaeal isolates. This is the first time cross-domain inhibition has been shown to occur in the environment, and this site will serve as an excellent model to study environmental chemical warfare among the three domains.

### 5.2.4 Common Features of Halocins

Halocins are either peptide (≤ 10 kDa; "microhalocins") or protein (>10 kDa) antibiotics produced by members of the archaeal family *Halobacteriaceae*. With one exception, halocin genes are induced at the transition between exponential and stationary phases (halocin H1 is induced during exponential phase; Platas et al. 1996). All

- halocin genes are located on megaplasmids (aka "mini-chromosomes"),
- halocin genes have typical haloarchaeal TATA boxes and TFB recognition elements (BRE), although the TATA box element for halocin C8 is a bit closer to the start site of transcription than usual (18 bp rather than 22–25 bp; Sun et al. 2005),
- halocin transcripts are "leaderless", where the transcriptional start site is either coincident with or only a few bps upstream of the translational start codon ATG,
- halocin preproteins appear to be exported by the twin arginine translocation (Tat) pathway, as all have a Tat signal motif at their amino terminus,
- mature halocins are inactivated by one or more proteases, confirming their proteinaceous nature,
- microhalocins are hydrophobic and are robust, as they can be desalted without losing activity, are insensitive to organic solvents such as acetonitrile and acetone, are relatively insensitive to heat (halocin R1 is the most sensitive, but can withstand heating at 60 °C for 1 h without losing activity; Table 5.1; O'Connor 2002), and can be stored at 4 °C for prolonged periods (as long as 7 years for halocin R1; O'Connor 2002) without significant loss of activity, and
- protein halocins (halocins H1 and H4) are heat-labile and lose activity when desalted below 5% (w/v) NaCl, although halocin H4 can be desalted to 10 mM $Na^+$ with only a twofold loss in activity (Perez 2000; Table 5.1). However, desalting to this level decreases the length of time halocin H4 can be stored at 4 °C.

### 5.2.5 Microhalocins (≤ 10 kDa)

#### *5.2.5.1 Halocin S8*

Halocin S8 (HalS8), produced by the uncharacterized haloarchaeal strain S8a isolated from the Great Salt Lake, UT by Penny Amy, was the first microhalocin to be characterized at both the protein and genetic levels (Price and Shand 2000). The mature microhalocin is composed of 36 amino acids with a molecular mass of 3.58 kDa. HalS8 contains four cysteine residues, which may form two disulfide bridges. However, no information on the tertiary structure of this microhalocin (or any other halocin, for that matter) is available. Currently, there is no evidence that HalS8 undergoes any post-translational modification of the amino acid sequence, but this has yet to be verified. The *halS8* gene is composed of a 933-bp open reading frame, yielding a 311 amino acid preproprotein upon translation. Processing of the preproprotein yields three separate proteins or peptides: a 230 amino acid N-terminal protein containing a typical Tat signal sequence, a 45 amino acid C-terminal peptide, and in between, the 36 amino acid mature halocin. Liberation of the halocin from the interior of its preproprotein is unique. Whether its release is autocatalytic or due to a protease is unknown (see De Castro et al. 2006 for a review of haloarchaeal proteases). Price and Shand (2000) speculated that the 230 amino acid N-terminal protein and the 45 amino acid C-terminal peptide might play roles in halocin immunity, regulation, induction, and/or translocation. However, BLAST searches revealed no matches to any other sequence within the database that would help to elucidate their possible function(s).

#### *5.2.5.2 Halocin R1*

Halocin R1 (HalR1), the second microhalocin to be characterized, is produced by *Hbt. salinarum* GN101, originally isolated from a solar saltern in Guererro Negro, Mexico by Barbara Javor (Ebert et al. 1986). Initial studies found HalR1 to have a molecular mass of 6.2 kDa by SDS-PAGE (Rdest and Sturm 1987). In contrast, later experiments revealed that halocin R1 appeared to be attached to a "carrier" protein, giving an apparent mass of about 29 kDa by gel filtration during purification (Shand et al. 1999; O'Connor 2002). However, upon heating the halocin-laden material prior to gel filtration, the halocin dissociated from the "carrier" protein and eluted at its true mass of 3.8 kDa. The HalR1 peptide consists of 38 amino acids, as determined by Edman degradation, with striking similarity to HalS8: HalR1 is 63% identical (capitalized residues below) and 71% similar to HalS8 (Price and Shand 2000; O'Connor 2002; O'Connor and Shand 2002):

- HalR1: `lqsNINiNTAAaVILiFNQVqvgALCaPTpVsGGgPpP`
- HalS8: `sdcNINsNTAAdVILcFNQVgscALCsPTIV-GG-PvP`

The small, yet significant differences in the amino acid sequences of these two microhalocins must be responsible for the differences in activity spectra and thermolability (Price and Shand 2000; O'Connor 2002; O'Connor and Shand 2002), but exactly which residues are involved remains to be determined.

Rdest and Sturm (1987) demonstrated that HalR1 is archaeostatic, as no changes in optical density or cell morphology of sensitive *Hbt. salinarum* (formerly *Hbt. halobium*) cells were noted after incubation with HalR1 for 7 days, and the cultures were able to resume growth upon removal of the halocin. Additionally, no zones of inhibition were seen when HalR1 was spotted onto fully grown lawns of sensitive cells, demonstrating that HalR1 is not archaeolytic. The archaeostatic response is dose-dependent, as increasing amounts of HalR1 resulted in proportional increases in the degree of inhibition, determined by the effect on growth in broth (Rdest and Sturm 1987).

#### *5.2.5.3 Halocin H6/H7*

Halocin H7 (HalH7, formerly known as HalH6) is produced by *Hfx. gibbonsii* Ma2.39, originally isolated from a solar saltern near Alicante, Spain (Torreblanca et al. 1986). The molecular mass of halocin H7 was initially calculated to be 32 kDa by gel filtration (Torreblanca et al. 1989). Similar to halocin R1, denaturing conditions (in this case, SDS-PAGE) released the mature halocin from a larger "carrier" protein, yielding a peptide of approximately 3 kDa (I. Meseguer, personal communication). Therefore, this halocin is now reclassified as a microhalocin. Although the size of the protein has been elucidated, the gene and protein sequences unfortunately are proprietary. Stability studies have shown that HalH7 can be desalted and is heat-resistant, which is consistent with the physicochemical stability profile of the other microhalocins (see Sect. 5.2.4 and Table 5.1). Halocin H7 is archaeolytic, described as having "single-hit kinetics" (a linear, inverse relationship between survival of sensitive cells and halocin concentration; O'Connor and Shand 2002) in the range of 5–80 arbitrary units (AU)/ml (Torreblanca et al. 1989). Exposure of sensitive cells to HalH7 caused the cells to swell and eventually lyse, indicating that the target site of activity of HalH7 is the cell membrane (Torreblanca et al. 1989). Further studies examined the effect of HalH7 on changes in intracellular volume, internal pH, membrane potential, proton motive force, and ionic flux in sensitive cells; results showed that the specific target of HalH7 is the $Na^+/H^+$ antiporter (Meseguer et al. 1995). This is significant, as it not only provides the first specific mechanism of action that can be attributed to any halocin, but it has also been shown to inhibit both haloarchaeal and mammalian $Na^+/H^+$ antiporters (Meseguer et al. 1995; Alberola et al. 1998; see Sect. 5.3).

#### *5.2.5.4 Halocin A4*

Halocin A4 is produced by an uncharacterized haloarchaeon isolated from a Tunisian saltern by Felicitas Pfeifer. It has been purified from a concentrated culture supernatant, using gel filtration column chromatography and reversed-phase HPLC as described in Shand (2006). The molecular mass of halocin A4 is 7,435 Da, as determined by mass spectrometry (very similar to halocin C8; see Sect. 5.2.5.5), and it is both acidic (pI = 4.14) and hydrophobic (eluting at ~85% acetonitrile from a reversed-phase column; Duncan 2004). It is characterized as having a "broad" spectrum of activity when challenged against other haloarchaeons (Table 5.1) but significantly, it also kills the crenarchaeal hyperthermophile *S. solfataricus* (Haseltine et al. 2001). *Sulfolobus solfataricus* mutants resistant to halocin A4 have been isolated (Haseltine et al. 2001), suggesting that there may be a common archaeal-specific target site shared by *Sulfolobus* and haloarchaeal cells sensitive to this halocin.

#### *5.2.5.5 Halocin C8*

Groundbreaking discoveries in the halocin field have been made by studying various aspects of halocin C8 produced by *Halobacterium* strain AS7092, isolated from the Great Chaidan Salt Lake, China (Li et al. 2003; Sun et al. 2005). It is the largest member of the microhalocin family (7.44 kDa, 76 amino acids) and is cysteine-rich, containing 10 cysteine residues. Halocin C8 is processed from the C-terminal end of a 283 amino acid preproprotein (called ProC8). The amino terminus contains a Tat leader sequence followed by a 207 amino acid, hydrophilic protein that confers immunity (called HalI). These are the first examples of both halocin immunity, and of an immunity protein and an antimicrobial peptide encoded in a single gene. In vitro, both unprocessed ProC8 and HalI containing the Tat leader sequence conferred immunity. HalI is associated with the membrane fraction of *Halobacterium* strain AS7092 (anchored by the Tat sequence?), and is thought to function by sequestering HalC8. In addition, heterologous expression of the gene sequence encoding HalI (named *halI* and under control of the bacterio-opsin promoter) in the HalC8-sensitive strain *Har. hispanica* also conferred immunity.

### 5.2.6 Protein Halocins (> 10 kDa)

#### *5.2.6.1 Halocin H1*

Halocin H1 (HalH1) is produced by *Hfx. mediterranei* M2a (formerly strain Xia3), originally isolated from a solar saltern near Alicante, Spain

(Rodriguez-Valera et al. 1982). It is a 31 kDa protein that is heat-labile (Platas 1995; Platas et al. 1996) and requires a minimum of 5% (w/v) NaCl to retain activity (Platas et al. 2002). Platas et al. (1996) determined that the nutrient source contained within the growth medium was the most important parameter influencing halocin production; growth in N-Z amine E yielded 1,280 AU/ml of halocin activity, while all other nutrients tested resulted in lower halocin production, ranging from 0–320 AU/ml of activity. The specific mode of action of HalH1 is unknown, but it appears to affect membrane permeability of sensitive cells (Platas 1995).

#### *5.2.6.2 Halocin H4*

Halocin H4 (HalH4) is produced by *Hfx. mediterranei* R4 (ATCC 33500), also originally isolated from a solar saltern near Alicante, Spain (Rodriguez-Valera et al. 1982). It was the first halocin discovered (Rodriguez-Valera et al. 1982), and has been fully characterized at both the protein and genetic levels (Meseguer and Rodriguez-Valera 1985, 1986; Cheung et al. 1997; Perez 2000). The molecular mass of HalH4 initially was determined to be approximately 28 kDa, using gel filtration and SDS-PAGE (Meseguer and Rodriguez-Valera 1985). However, once the *halH4* gene was cloned and the amino acid sequence determined by Edman degradation, the molecular mass of the mature HalH4 protein was calculated to be 34.9 kDa (359 amino acids), processed from a preprotein of 39.6 kDa (Cheung et al. 1997). The preprotein contains a 46 amino acid N-terminal Tat signal sequence (atypically long; Eichler 2000) important in translocation of the protein across the membrane (Cheung et al. 1997). How, when, and where the signal sequence is removed from the preprotein is unknown. The mature halocin also contains a 32 amino acid hydrophobic region in the middle of the protein sequence, which may be functionally important (e.g., in binding to the target site; Shand et al. 1999). The *halH4* gene consists of a 1,077-bp open reading frame encoding the 359 amino acid preprotein (Cheung et al. 1997). Cheung et al. (1997) concluded that expression of the *halH4* gene, in addition to being regulated at the level of transcription, must also be regulated post-transcriptionally. Halocin H4 is an archaeolytic halocin, described by Meseguer and Rodriguez-Valera (1986) as having "single-hit kinetics" similar to halocin H6/H7 (see Sect. 5.2.5.3). Halocin H4 adsorbs to sensitive *Hbt. salinarum* cells where it appears to disrupt membrane permeability, resulting in an ionic imbalance and leading to cell lysis. Examination of halocin activity showed sensitive cells became swollen and spherical in the presence of HalH4 (Meseguer and Rodriguez-Valera 1986), indicating that its primary target is localized in the membrane (Rodriguez-Valera et al. 1982; Meseguer and Rodriguez-Valera 1986). However, experiments to elucidate the specific target site have not revealed the actual target (Meseguer et al. 1995).

## 5.3 Biotechnology of Halocins

The potential of halocins as chemotherapeutic agents active against human or animal pathogens has been unrealized, but is potentially vast, given the hundreds of different halocins reported to exist versus the number of halocins actually characterized. Halocin H7, however, has been shown to inhibit the $Na^+/H^+$ antiporter (aka "exchanger") in both haloarchaea (Meseguer et al. 1995) and in a dog model (Alberola et al. 1998). The latter is significant, in that this halocin may serve as treatment to reduce injury caused when ischemic transplanted organs are reperfused (e.g., by reducing infarct size and the number of ectopic beats in a heart transplant; Alberola et al. 1998). The basis of this biomedical application was the discovery of the mechanism of action of halocin H7. Consequently, applications for other halocins will also hinge on the discovery of their mechanisms of action.

The *halI* gene may serve as a useful selectable marker especially for haloarchaea that require the highest levels of NaCl for optimal growth (Sun et al. 2005). Similarly, if the *S. solfataricus* gene that carries a mutation for resistance to halocin A4 can be isolated, it too might serve as a selectable marker for these crenarchaeal hyperthermophiles (Haseltine et al. 2001; O'Connor and Shand 2002).

## 5.4 Sulfolobicins

The archaeocins produced by *Sulfolobus* are entirely different from halocins, since their activity is predominantly associated with the cells and not the supernatant (Prangishvili et al. 2000). Prangishvili et al. (2000) were the first to isolate and characterize these proteinaceous toxins, which they called "sulfolobicins", in keeping with bacteriocin nomenclature. Provisionally, the producer strain has been named "*Sulfolobus islandicus*". Screening for sulfolobicin activity involves spotting samples of exponentially growing "*S. islandicus*" cells onto lawns of the sensitive strain *S. solfataricus* P1. Following incubation, nearly clear zones with sharp borders are generated, the size of the zone of inhibition being inversely proportional to the concentration of sensitive cells in the lawn. To date, the spectrum of sulfolobicin activity appears to be restricted to other members of the sulfolobales: the sulfolobicin inhibited *S. solfataricus* P1, *S. shibatae* B12, and six non-producing strains of "*S. islandicus*". Activity appears to be archaeocidal but not archaeolytic. It does not inhibit *S. acidocaldarius* DSM639, nor does purified sulfolobicin from strain HEN2/2 inhibit *Hbt. salinarum* R1 or *Escherichia coli* (Prangishvili et al. 2000).

Unlike halocins, sulfolobicins are not secreted into the culture medium in any significant quantity, and classical inducing agents (UV light, temperature and pH shifts, and exposure to sensitive cells) used to increase secretion have not been successful (Prangishvili et al. 2000). Analysis of sulfolobicin activity

in a 500 ml culture revealed that 30 times more activity can be purified from the cell pellet than from the culture supernatant. To visualize activity in culture supernatants, the supernatant from stationary phase cultures had to be concentrated 100-fold, either by precipitation or centrifugation, before any activity was detected when spotted onto a lawn of sensitive cells.

Extracellular activity is associated with spherical particles 90 to 180 nm in diameter. These particles are present in a ratio of 1:100 cells, and are also produced by strains that do not make sulfolobicin. When purified using CsCl density gradient centrifugation, these particles form a discrete band with a density of approximately 1.29 g/ml. Electron micrographs of this material revealed an inner core with a surrounding layer having a periodicity of 22 nm, the same as the lattice constant of the *Sulfolobus* S-layer (Prangishvili et al. 2000).

Purification of sulfolobicin involves harvesting cells from late stationary phase, sonicating them, collecting the resultant cell ghosts by high-speed centrifugation, and releasing the sulfolobicin with Triton X-100. Activity elutes in the range of 30 to 40 kDa on size exclusion chromatography, in contrast to 20 kDa on SDS-PAGE. These data suggest that this archaeocin may aggregate (Prangishvili et al. 2000). Activity of purified sulfolobicin remains stable after 6 months at 4 °C or 5 days at 85 °C. Enzymatic treatment with α-amylase, α- and β-glucosidases, phospholipase C, and lipoprotein lipase had no effect on activity. However, treatment with pronase E, proteinase K, and trypsin completely destroyed activity, indicating activity is associated with a proteinaceous component (Prangishvili et al. 2000).

Sulfolobicins exhibit some classical bacteriocin characteristics, as they are proteinaceous and are directed against strains that are closely related to the producer. Although some of the producer strains contain conjugative plasmids, neither sulfolobicin production nor immunity can be transferred to non-producer strains, suggesting that the genes for these traits may be located on the chromosome. Although evidence suggests that sulfolobicins remain bound to cells or associated with S-layer-coated vesicles, it does not exclude the possibility that an undetectable amount of sulfolobicin may leak out from cells or vesicles into the surrounding medium. Indeed, such a scenario could account for the generation of large zones of inhibition on solid medium where the concentration of free sulfolobicin would remain more localized and high. This phenomenon also is seen with cell-bound bacteriocins (Prangishvili et al. 2000).

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