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Halocins and sulfolobicins: The emerging story of archaeal protein and peptide antibiotics

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Production of antibiotic peptides and proteins is a near-universal feature of living organisms regardless of phylogenetic classification. Bacteriocins (proteinaceous antimicrobials from the domain Bacteria) have been studied for over 75 years, and the eucaryocins (proteinaceous antimicrobials from the domain Eucarya) since the early 1960s. However, one domain of organisms, the Archaea, containing hyperthermophiles, extreme halophiles and the methanogens, is just beginning to be scrutinized for the production of peptide antibiotics. Production of archaeal proteinaceous antimicrobials (archaeocins) from extreme halophiles (halocins) is a nearly universal feature of the rod-shaped haloarchaea. Halocin activity is first detectable in culture supernatants at the beginning of the transition into stationary phase, concomitant with an induction of transcription of the structural gene. Halocins are diverse in size, consisting of proteins as large as 35 kDa and peptide ''microhalocins'' as small as 3.6 kDa. The 36 amino acids of microhalocin HalS8 are located in the interior of a 311-residue pro-protein from which they are liberated by an unknown mechanism. Microhalocins are hydrophobic and robust, withstanding heat, desalting and exposure to organic solvents. Unlike the peptide bacteriocins and the eucaryocins, microhalocins possess a large number of neutral residues and are not cationic, leaving their mechanism(s) of action mostly a mystery. While microhalocins affect a variety of haloarchaeal genera (kingdom Euryarchaeota), they also exhibit cross-kingdom toxicity, inhibiting or killing *Sulfolobus* species (kingdom Crenarchaeota). Finally, archaeocins also are produced by the hyperthermophile ''*Sulfolobus islandicus*''. These 20-kDa protein antibiotics are not excreted into the environment, but are associated with small particles apparently derived from the cell's S-layer.
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Introduction

The bacterial production of substances antagonistic to other bacteria has been known since 1925, beginning with the discovery of antagonism between strains of *Escherichia coli* [13]. Originally, these first substances were given the name ''colicins,'' but now gene-encoded antimicrobial proteins and peptides produced by members of the domain Bacteria are known as bacteriocins. In the early 1960s, the study of animal antimicrobial peptides began in earnest, and in the early 1980s, the first structures were elucidated: a cecropin from an insect and a defensin from a mammalian phagocyte [4]. Since then, numerous antimicrobial peptide antibiotics from eucaryotes have been described from sources now including frogs, birds, protozoans and plants, in addition to mammals and insects. In 1982, proteinaceous antimicrobial compounds from several extremely halophilic members of the domain Archaea (''halocins'') were discovered [52]. Until recently, antimicrobial proteins and peptides in this domain were limited to the extreme halophiles, when a proteinaceous antimicrobial compound, a ''sulfolobicin,'' was described from the hyperthermophilic crenarchaeote ''*Sulfolobus islandicus*'' [46].

Peptide antibiotics can be synthesized in two different ways: ribosomally from transcripts (gene encoded), or by stepwise synthesis employing either multienzyme complexes or sequential enzyme reactions [26]. Familiar examples of nonribosomally synthesized peptide antibiotics include gramicidin (a cyclic decapeptide), bacitracin (an undecapeptide) and valinomycin (a cyclododecadepsipeptide). This review is limited to ribosomally synthesized protein or peptide antimicrobials. The size boundary between a protein and a peptide overlaps and is somewhat arbitrary. The smallest compound to be called a protein is around the size of insulin (~5800 Da), while the largest peptide is rarely above 10 kDa [4]. Size plays an important role in the classification of all proteinaceous antimicrobials since both peptide and protein antibiotics are found in all three domains.

We have adopted the three-domain classification system proposed by Woese *et al* [66] as an overarching framework for classifying all ribosomally synthesized proteinaceous antimicrobials: bacteriocins are produced by members of the domain Bacteria, archaeocins [47] are produced by members of the domain Archaea and eucaryocins are produced by the members of the domain Eucarya. These distinctions are especially important for proteinaceous antimicrobials from the domains Bacteria and Archaea. While members of the Archaea are ''prokaryotic'' in cellular organization, they should not be confused phylogenetically with organisms in the domain Bacteria. Consequently, calling archaeal proteinaceous antimicrobials ''bacteriocins'' is misleading and inappropriately groups the Archaea with the Bacteria [47].

Table 1 summarizes the salient features of the halocins that have been described to date followed by descriptive reviews of the more highly characterized halocins. Halocin designations are alphanumeric (e.g., halocin H4) and carry over to their protein (HalH4) and gene (*halH4*) designations. In parallel with the nomenclature for microcins, peptide halocins (~4–5 kDa) are called ''micro-

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Table 1 Halocin characteristics

Halocin	Producer (source)	Size (kDa)	Thermal stability	Salt-dependent	Activity spectrum[a]	Mechanism	Refs.
A4	Strain TuA4 (solar saltern, Tunisia)	<5	≥1 week at boiling[b]	No	Broad *Sulfolobus* spp.	ND	[17] (Kemper S and R Shand, unpublished)
G1	*Halobacterium* strain GRB (solar saltern, France)	ND[c]	ND	ND	Broad	ND	[59]
H1	*Hfx. mediterranei* Xai3 (solar saltern, Spain)	31	<50°C	Yes	Broad	Membrane permeability	[44,45,52]
H2	Strain GLA22 (solar saltern, Spain)	ND	ND	ND	Broad	ND	[52]
H3	Strain GAA12 (solar saltern, Spain)	ND	ND	ND	Broad	ND	[52]
H4	*Hfx. mediterranei* R4 (solar saltern, Spain)	39.6 (preprotein) 34.9 (mature)	<60°C	Partially[d]	Narrow	Proton flux?	[8,33,34,36,43,52,56]
H5	Strain MA220 (solar saltern, Spain)	ND	ND	ND	Narrow	ND	[52]
H6/H7	*Hfx. gibbonsii* Ma2.39[e] (solar saltern, Spain)	32	≤90°C	No	Narrow	Na^+/H^+ antiporter inhibitor	[1,36,52,63]
R1	*Halobacterium* strain GN101 (solar saltern, Mexico)	3.8	60°C	No	Broad *Sulfolobus* spp. *M. thermophila*	ND	[17,48,56] (O'Connor E and R Shand, unpublished)
S8	Strain S8a (Great Salt Lake, UT)	33.9 (proprotein) 3.6 (mature)	≥24 h at boiling[b]	No	Broad *Sulfolobus* spp.	ND	[17,47,56]

[a]Activity spectrum refers to inhibition of haloarchaea, unless otherwise indicated.
[b]This study was done at 2113 m (7000 ft); water boils at 93°C at this elevation.
[c]ND, not determined.
[d]See text.
[e]Halocin H6 is produced by *Hfx. gibbonsii* Ma2.39. This strain 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.

halocins'' [47]. Since not all haloarchaea are sensitive to any particular halocin, a ''sensitive'' strain is one in which a zone of inhibition appears on a double-agar overlay plate in response to the presence of the halocin. A brief overview of peptide bacteriocins and review articles describing the various types of eucaryocins follow and serve as a preface for the discussion on halocins and sulfolobicins.

Bacteriocins

Peptide and protein antibiotics produced by members of the domain Bacteria are numerous, and are divided into three categories: colicins and colicin-like proteins, peptide antibiotics made by Gram-positive organisms and small peptides produced by Gram-negative organisms called ''microcins.'' The field of colicins and colicin-like proteins is vast, and we point the reader to the following reviews: general aspects [6,25,58], channel formation [2,22,61], import [27], ecology [51] and evolution [50]. Brief descriptions of peptide antibiotics made by Gram-positive organisms and of microcins follow.

Peptide antibiotics from Gram-positive bacteria

Gram-positive bacteria produce an extensive array of ribosomally synthesized peptide antibiotics that differ widely in both the degree and type of post-translational modification they possess. The vast majority of these peptides are small (2–6 kDa) and heat-stable, although there are some examples of larger (>30 kDa), heat-labile proteins. Except for the class IB lantibiotics and some large proteolytic bacteriocins, the bacteriocins from Gram-positive bacteria are generally cationic and form amphipathic α-helices in the presence of anionic phospholipids. Their cationic nature facilitates nonspecific interactions with target Gram-positive bacteria, which results in the formation of ion channels or pores. In contrast, class IB lantibiotics are only about 20 amino acids long, have little or no negative charge, are rigidly globular and inhibit specific enzymes in the target cell. Small Gram-positive bacteriocins are generally divided into two classes: those that contain the two novel amino acids lanthionine and methyllanthionine (called lantibiotics or class I bacteriocins), and those that do not (called nonlantibiotics or class II bacteriocins).

Lantibiotics (reviewed in Refs. [3,14,20,21,38–40]): Lanthionine, methyllanthionine and other unusual amino acids found in lantibiotics are generated post-transcriptionally and confer vast structural diversity to this class of molecules. Lantibiotics can be subdivided into class IA, consisting of elongated 2–5 kDa molecules that interact with target cell membranes to form voltage-dependent pores, and class IB, immunologically active globular molecules that inhibit specific enzymes. Both classes of lantibiotics make use of ''docking molecules'' on the cell surface in order to

bind to target cells, although the way they use docking molecules may differ.

Nonlantibiotics (reviewed in refs. [3,16,21,38–40]): Class II bacteriocins (or nonlantibiotics) are small (<10 kDa), unmodified, heat-stable peptides that are divided into four subgroups, as described below.

Class IIA: pediocin-like, or *Listeria*-active bacteriocins. Class IIA bacteriocins, such as pediocin PA-1 from *Pediococcus acidilactici*, form pores and share 40% to over 70% homology, with the highest degree of conservation in the hydrophilic, cationic N-terminal "pediocin box" region. The N-terminal regions of these molecules are thought to be responsible for initial interactions with the target cell membrane, while hybrid molecule studies indicate that the more variable, hydrophobic, often amphiphilic C-terminal end of the molecule is responsible for pore formation and target cell specificity.

Class IIB: two-peptide bacteriocins. A defining characteristic of class IIB bacteriocins is very weak or missing biological activity when the component peptides are tested individually, and synergy when tested in concert. The peptide pairs of class IIB bacteriocins range in size from 25 to 62 amino acids and are thought to have no shared ancestry, since with the exception of a few cases, they generally share no sequence homology within the pair or with other class IIB bacteriocins. Given the weak activity that class IIB bacteriocins exhibit as individual peptides, their synergistic interaction and their usual lack of sequence relatedness, it is generally thought that this class of pore-forming bacteriocins is derived from individual bacteriocins that evolved over time to act cooperatively to form a more efficacious toxin.

Class IIC: *sec*-dependent bacteriocins. Class IIC bacteriocins are a structurally and functionally heterogeneous family of peptides that are grouped together solely on the basis of the presence of a *sec*-dependent signal sequence, with disregard for other structural characteristics they may share with other class II bacteriocins. In addition to enterocin P, a pore-forming bacteriocin with a sequence similar to a pediocin box, this group also includes lysostaphin, a peptidoglycan endopeptidase.

Class IID bacteriocins. Class IID bacteriocins are, by their very definition, a heterogeneous group of peptides, since they are comprised of all other class II bacteriocins that do not fit structurally into classes IIA, B and C.

Microcins (reviewed in Refs. [3,20,40])
Gram-negative bacteria, in particular the Enterobacteriaceae, produce small (<20 kDa), ribosomally synthesized peptide antibiotics called microcins. These peptides may be unmodified or may possess a wide array of post-translational modifications, including multiple heterocycles, head-to-tail cyclization, formylation and the addition of modified nucleic acid bases. Microcins have been subdivided into three classes based on their mode of action: class A microcins inhibit metabolic enzymes, class B microcins inhibit DNA replication and class D microcins interfere with energy generation.

Class A microcins: Microcin C7 (MccC7) is a heptapeptide that contains an N-terminal formylated methionine, a C-terminal modified nucleic acid base and inhibits protein synthesis in the Enterobacteriaceae [12,15]. MccC7 is the product of the smallest known gene, which consists of a 21-base open reading frame with a ribosome binding site six bases upstream from the start of translation [12]. Analog studies suggest that the C-terminal substituent is responsible for transport, and that the peptide itself is responsible for inhibitory activity [15].

Class B microcins: Microcin B17 (MccB17) inhibits DNA gyrase and is an example of a glycine-rich, highly modified class B microcin. MccB17 is synthesized as a 69-amino-acid precursor with a 26-amino-acid leader that is cleaved prior to the formation of the thiozole and oxazole rings that are responsible for its activity.

Class D microcins: ColV, which inhibits proline transport and the generation of membrane potential in *E. coli*, and microcin E492 (MccE492) from *Klebsiella pneumoniae*, which destroys membrane potential by forming pores, are examples of 6 kDa unmodified class D microcins. Originally identified as a colicin, ColV is more properly classed as a microcin, since it is small and does not have the properties of a colicin. MccE492 is unusual for a bacteriocin in that it is the only microcin to date that is produced maximally during exponential growth, and its structural and immunity genes are thought to be on the chromosome. At stationary phase, there is also the production of a microcin antagonist that modulates its activity [65].

Eucaryocins

Protein and peptide antimicrobials from organisms in the domain Eucarya have been studied since the early 1960s, and the field is now vast. Recent publications include a conference report [4], protocol methods [54], general reviews [5,31], an excellent review of antimicrobial peptides based on their biochemistry and structure [10], an excellent historical perspective of peptide antibiotics by Spitznagel [60] and thousands of monographs. There is also a database of peptide antibiotics maintained by Alessandro Tossi that contains more than 600 sequences from plants and animals (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm).

Just as bacteriocins are produced by a wide variety of bacteria, eucaryocins have been found in mammals (reviewed in Refs. [11,28–30,32]], frogs (reviewed in Ref. [57]), horseshoe crabs (reviewed in Refs. [18,19,28]), insects (reviewed in Ref. [42]), protozoans (reviewed in Ref. [67]) and plants (reviewed in Ref. [7]). In animals, they represent an important part of an animal's innate immunity (reviewed in Ref. [5]). Typically, but by no means universally, eucaryocins are small, heat-resistant cationic peptides, frequently cysteine-rich, that form β-sheets or amphipathic α-helices, and act by disrupting the target membrane in some fashion. In parallel with bacteria, it is clear that antimicrobial peptides are produced by nearly every eucaryotic organism examined.

Archaeocins (halocins and sulfolobicins)

Ubiquity of halocin production and the significance of halocins in the environment

Two nonoverlapping antagonism studies employing 79 and 68 different haloarchaeal isolates representing 12 and 9 taxonomic groups (phenons), respectively, have been reported [35,64]. Both studies were performed in an essentially identical fashion by challenging each strain against all other strains as well as against itself. The presence of zones of inhibition on double-layer agar plates was used as the indicator for antagonism. Both studies showed that phage production was never the cause of the inhibition, and both demonstrated that the antagonism was protein-based by eliminating or reducing inhibition after treatment with proteases. The results of the two studies were consistent: (i) the activity spectrum varied widely among the different halocins — some inhibited nearly all the strains; others inhibited only a few; (ii) only 3 of 147 strains showed no sign of producing inhibitory activity; (iii) based on similarities among the spectra, the first study placed 48/79 producers into 15 different groups, while the second study placed 35/68 producers into 8 groups; (iv) of the 144 producers, 20 were sensitive to their own halocin and (v) none of the isolates was completely insensitive to all halocins. Taken together, these two studies indicate that there are numerous classes or groups of halocins, and that "... halocin production is a practically universal feature..." of the haloarchaea [64]. This conclusion is identical to that stated by Tagg [62] in 1992 for bacteria: given a sufficient number of indicator strains and the proper testing conditions, bacteriocin production will be a universal feature.

Given their ubiquity, the main teleology for the existence of bacteriocins and halocins has always been that they reduce competition [6,41,56]. Moreover, proteinaceous antimicrobials that can lyse competitors enrich the environment for the producer. For example, Platas *et al.* [45] found that in a co-culture of *Haloferax mediterranei* (a halocin producer) and *Halobacterium salinarum* (a nonproducer) that was devoid of any nutrients, *Hfx. mediterranei* was able to grow at the expense of *Hbt. salinarum* (presumably through release of cellular constituents from *Hbt. salinarum*). Intrigued by the observation that hypersaline lakes and crystallizer ponds tend to be dominated by one or a few haloarchaeal genera, Kis-Papo and Oren [24] tried to determine if halocins were a factor in this observation, and what ecological role, if any, they might play in haloarchaeal diversity in the field. Twelve brine samples were obtained from four crystallizers: two from Israel, one from Spain and one from California, USA. Direct bacterial cell counts at the time of sampling ranged from 8.4×10^6 to 7.2×10^8 cells/ml, with cell densities typically between 10^7 and 10^8 cells/ml. Using 12 indicator organisms, no halocin activity was found in any of the cell free filtrates, even after concentration by ultrafiltration. Kis-Papo and Oren point out that potential halocin activity might be reduced by proteolytic degradation or by adherence of the halocin to the ultrafilters (although activity in filtrates containing a known halocin was only reduced two-fold due to the ultrafiltration process). Consistent with these findings is the observation that halocin activity is reduced or disappears when mixed with indicator cells [33,52] (Dugas S and R Shand, unpublished). In a report on halocin H4 [33], it was observed that a concentrated supernatant lost half of its activity after 24 h of exposure to sensitive cells. It is not known if this experiment was done under saturating conditions. In preliminary experiments with other halocins, where target sites may or may not have been saturating, we have shown that halocin A4 activity disappeared immediately upon mixing (in 1 min or less), halocin R1 activity began to diminish after 5 min (but some activity still remained after 24 h) and halocin S8 activity did not seem to diminish at all (Dugas S and R Shand, unpublished). A major contributing factor to the absence of detectable halocin activity in the environment may be that externalized halocin molecules adsorb quickly to sensitive cells or even to cellular debris, rendering them inactive and undetectable.

Halocin H1

Halocin H1 is a 31-kDa protein produced by *Hfx. mediterranei* Xia3 [45]. It is thermolabile above 50°C and salt-dependent, denaturing below salt concentrations less than 10% (w/v) [45]. It has a broad spectrum of activity with respect to other haloarchaea [35] and a mechanism of action involving alteration of membrane permeability [44]. Platas *et al.* [45] optimized conditions for maximal production of this halocin [20% (w/v) salts, 0.5% (w/v) N-Z amine E, 37°C under aeration, ~6 h generation time] and found that the critical parameters were salt concentration, temperature and, most importantly, nutrient source. Halocin activity in stationary phase ranged from 0 to 1280 arbitrary units (AU) among the 13 nutrient sources examined, with N-Z amine E as the superior nutrient. Factors that had no effect included oxygen tension, light, inoculum size and enriching the medium with other nutrients. Halocin activity is first detectable during mid-exponential phase instead of at the onset to stationary phase, as is seen with most other halocins. (Note that in the halocin field, the term "arbitrary unit" is typically defined as the reciprocal of the dilution at which antibiotic activity disappears, i.e., the reciprocal of the extinction dilution [56]. Across fields, however, there is no agreed upon method used to quantify activity levels, and the method used by an individual investigator is described within the manuscript.)

Halocin H4

Halocin H4 from *Hfx. mediterranei* R4 (ATCC 33500) was the first halocin to be studied [52] and is now well characterized [8,33,34,43]. Samples with high levels of activity produce large, clear zones on top agar overlays of sensitive cells. The zones produced from samples with lower activities are often very turbid, and extinction dilutions are difficult to determine. Halocin H4 is a protein antibiotic with calculated molecular masses of 34.9 kDa (mature) and 39.6 kDa (pre-protein) from the sequence of the *halH4* gene [8]. However, there is a 7-kDa difference between the calculated mass of the mature protein and the mass determined from two nondenaturing (i.e., high salt) gel filtration studies using concentrated culture supernatants: ~28 kDa [33,43]. In studies done by Rodriguez-Valera (personal communication) and Perez [43], more-highly purified material gave molecular masses of 30 and 33.5 kDa, respectively, on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The discrepancy between the predicted mass and that obtained from gel filtration may be due, in part, to the high viscosity produced by solvents used in gel filtration studies (2.6 M [43] and 4.3 M salt [33]) and to the tertiary structure of this extremely halophilic protein.

Given its size, it is not surprising that halocin H4 is sensitive to proteases and heat [43,52]. The shelf-life of concentrated halocin H4-containing supernatants is between 115 and 159 days at 4°C. Perez showed that there is no loss of activity in culture supernatants at 51°C after 24 h of incubation. This is understandable, since 51°C

is an optimal growth temperature for *Hfx. mediterranei* [55]. However, activity is lost completely in 24 h at 60°C, 4 h at 70°C and in <30 min at 80°C and above. Rodriguez-Valera *et al.* [52] reported that halocin H4 activity was also lost when the salt concentration was reduced below 5% (w/v) by dialysis. Perez revisited this observation and found that halocin H4 could be desalted to 10 mM Na^+ using recursive ultrafiltration and then resalted, resulting in at most a two-fold loss in activity, but a greatly reduced shelf-life. This was an important finding since it allowed the use of ion exchange HPLC for purification [43].

The coding region of the *halH4* gene is 1077 bp (359 amino acids) and encodes the HalH4 pre-protein [8]. There is a 46-amino acid signal sequence that is absent from the mature, secreted protein. How, when and where the sequence is removed is unknown. The net charge (+6) of the N-terminal region is very similar to that found in the signal sequences from a halolysin [23] and from a xylanase from *Streptomyces halstedii* [53]. The gene is located on a 320-kb megaplasmid (pHM300), has a typical haloarchaeal TATA box promoter hexamer and the *halH4* transcript is leaderless with just four bases between the start site and the initiator AUG codon. Transcription is terminated with a stem-loop structure (11 basepair stem, 16 base loop). Expression of the *halH4* gene is regulated: Cheung *et al.* [8] have shown that in a complex medium, transcript levels increased six- to seven-fold between their low basal levels during exponential phase and their induced levels at the beginning of the transition to stationary phase. The mRNA levels remained elevated during the transition, and then returned to basal levels by the end of the transition. Halocin H4 activity levels in the culture medium spiked from undetectable to 128 AU, paralleling the induction spike of the *halH4* transcripts. However, once the maximum halocin activity level was reached, the level dropped rapidly four-fold to 32 AU, and remained at this level throughout stationary phase.

Identifying the mechanism of action of halocin H4 has been elusive. Meseguer and Rodriguez-Valera [34] found that over several hours of exposure, *Hbt. salinarum* CCM 2090 cells sensitive to this halocin became swollen and spherical, distinct changes in the cytoplasm appeared and the cells eventually lysed. They eliminated macromolecular synthesis, membrane pores, energy metabolism and the cell wall as primary targets. However, halocin H4 did decrease the rate of return of H^+ from the medium across the membrane to the interior. The two main systems involved in this transport are the membrane ATPase and the Na^+/H^+ antiporter: the ATPase was eliminated as the target since intracellular ATP levels were unaffected by halocin treatment; and in later studies, there was very little effect on Na^+/H^+ exchange in membrane vesicles, which eliminated the Na^+/H^+ antiporter as well [36].

Single-hit kinetics: Meseguer and Rodriguez-Valera [34] reported that killing of sensitive cells by halocin H4 followed "single-hit kinetics." A semilogarithmic plot of survivors *versus* halocin H4 concentration was linear, showing that the killing of a given concentration of sensitive cells was proportional to the concentration of the halocin. Does this mean that one halocin H4 molecule kills one sensitive cell? Not necessarily. This concept is nicely clarified in an early review of bacteriocins by Reeves [49]. Early work with several colicins showed that there was a proportional correlation between the amount of colicin and either the number of bacteria killed or the initial rate of killing. Since these results suggested that one colicin killed one bacterium, the term "single-hit kinetics" was born. Reeves [49] pointed out that there are two factors one must consider when analyzing these types of kinetic data:

> [F]irst, one must take into account the statistical difficulty of distinguishing between the one-hit and other low-order reactions when only the surviving bacteria (and not the killed) can be scored directly... [Second, t]he most that kinetic data alone can show is that death results from the interaction of a certain number of colicin molecules and one bacterium. Should the data suggest that one molecule kills, this does not imply that the adsorption of a colicin molecule has a probability of one of leading to cell death, but only that the probability is unaffected by the adsorption of more colicin molecules. Holland... has shown that more than 100 molecules of megacin are required to kill a cell; the discrepancy between this observation and the apparent first-order kinetics [for megacin] could, in fact, be explained if each molecule on adsorption had only [a] 1 in 100 probability of leading to cell death, owing to a constant probability of 1 in 100 for each interaction, to only 1 in 100 receptors being sensitive, or to only 1 in 100 molecules of his purified material being active.

Consequently, assigning numbers of molecules required to kill a sensitive cell cannot be determined solely from semilogarithmic plots of survivors *versus* antibiotic concentration, even if the killing kinetics are described as "single hit."

Halocin H6

Halocin H6 (HalH6) from *Hfx. gibbonsii* Ma2.39 was first isolated and described by Torreblanca *et al* [63] in 1989. The onset of detectable activity in culture supernatants is typical, occurring at the transition into stationary phase. Activity reaches maximal levels at stationary phase and then decreases gradually thereafter. Unlike colicins, production of HalH6 is not affected by medium composition, nor is production inducible by UV light or acridine orange. Efforts to correlate HalH6 production with the presence of a plasmid were unsuccessful, since the plasmid could not be cured from the strains. HalH6 activity is resistant to trypsin but sensitive to pronase, demonstrating the proteinaceous nature of this inhibitory factor [63].

HalH6 has been purified using hydroxylapatite chromatography, gel filtration on Sephadex G50 and HPLC on Spherogel TSK3000 SW. The molecular weight of HalH6 is 32 kDa by gel filtration and slightly less than 31 kDa by SDS-PAGE [63]. Despite the purification, protein and gene sequences are not yet available. HalH6 can be desalted and is remarkably heat-resistant: after 10 min at 90°C and 100°C, HalH6 retains 100% and 50% of its activity, respectively. Total destruction of activity is only achieved by autoclaving [63]. This kind of heat resistance is more typical of small peptide bacteriocins [21,39], eucaryocins (see above) and microhalocins HalS8 and HalR1 (see below), than for proteins with molecular masses in the 30-kDa range.

Exposure of sensitive cells to HalH6 causes the intracellular volume to increase. Cellular swelling is followed by lysis, suggesting that HalH6 may act at the level of the cell membrane [63]. Following this lead, experiments that measured changes in cell volume, internal pH, membrane potential, proton motive force and sodium and proton flux in response to HalH6 were conducted and showed that the Na^+/H^+ antiporter is the target of this halocin [36].

Halocin S8, the first example of a microhalocin

Halocin S8 (HalS8) is the product of haloarchaeal strain S8a, isolated from the Great Salt Lake, UT [47]. This is the first example of a microhalocin, consisting of only 36 amino acids and having a molecular weight of 3.58 kDa [47]. It has a narrow spectrum of activity with respect to the haloarchaea, inhibiting only *Hbt. salinarum* NRC817, *Halobacterium* sp. strain GRB and *Hfx. gibbonsii* [47]. Like HalH6, HalS8 can be desalted and is heat-resistant, retaining 100% of its activity after 1 h at 93°C [47,56]. In addition, HalS8 activity is resistant to organic solvents and remains unchanged after storage at 4°C for several months [47,56]. HalS8 activity is due to the presence of a proteinaceous substance, since it is resistant to trypsin but sensitive to proteinase K [47,56].

HalS8 has been purified by concentrating culture supernatants using tangential flow filtration with filters of successively smaller nominal molecular weight cutoffs, followed by size exclusion chromatography in high salt (>4 M) buffer and reversed-phase HPLC. Although only 4 kDa, an anomaly occurred during tangential flow filtration where activity partitioned equally between the 30-kDa filtrate and the 30-kDa retentate. SDS-PAGE of the 30-kDa retentate revealed the presence of numerous small proteins, suggesting that nonspecific binding to these larger proteins was responsible for the lack of correlation between the 30-kDa nominal molecular weight cut-off filter and the size of HalS8 [47,56].

Edman degradation of purified HalS8 revealed a 47% hydrophobic, 36-amino-acid peptide (see sequence below) [47]. HalS8 is about as hydrophobic as the average peptide bacteriocin and is cysteine-rich like many eucaryocins. However, unlike Gram-positive bacteriocins and most eucaryocins, HalS8 has an abundance of neutral polar residues and only two charged residues, both of which are negative. Nevertheless, sequence similarities were sought between HalS8 and other peptide antibiotics: BLAST searches of HalS8 revealed no homologues with any protein presently in the database [47]. At present, there is no evidence other than long-term stability that HalS8 possesses post-translational amino acid modifications, but further investigations are required to rule this out.

The gene for HalS8 has been cloned and sequenced, and contour-clamped homogeneous gel electrophoresis analysis shows that it is located on a ~200 kbp megaplasmid. Like many haloarchaeal genes, primer extension reveals that the start sites of transcription and translation are coincident with each other, resulting in synthesis of a leaderless transcript. Consistent with the haloarchaeal consensus sequence (5′-TTTWWW-3′), the *hal*S8 promoter (5′ATTTAT-3′) is located from −29 to −24 bp upstream from the start site of transcription and contains a transcription factor B recognition element upstream of the promoter at −34 and −35 [47].

HalS8 activity is undetectable in culture supernatants until the culture begins the transition into stationary phase [47,56]. Activity reaches a maximum within 10 h of onset and is stable for greater than 80 h after reaching maximum values [47]. The expression pattern of *halS8* is identical to that of *halH4*, except that HalS8 activity levels remain elevated and constant throughout stationary phase. Initial transcript levels parallel halocin activity, remaining at very low basal levels throughout exponential growth and increasing nine-fold in concert with the rise of halocin activity in the supernatant. Thirty hours after the onset of activity, transcript levels decline gradually to basal levels, while halocin activity remains unchanged. Northern blot analysis showed the presence of two major transcripts (approximately 1070 and 1210 bases with identical patterns of expression) and one minor transcript (960 bases). Analysis of the 3′ end of *halS8* uncovers only one weak haloarchaeal "T-tract" transcriptional termination sequence starting at position 1266, which corresponds to the largest of the three *halS8* transcripts. There is no evidence of any stem-loop terminators [47].

The sequence corresponding to HalS8 is contained within a 933-bp open reading frame that codes for a 33,962-Da protein. HalS8 is processed from the interior of this 311-amino acid proprotein, a process that is common in eucaryocins but not in peptide bacteriocins. Processing results in a 230-amino acid amino terminal protein and a 45-amino acid carboxy terminal peptide. The mechanism of HalS8 excision does not appear to be analogous to that of inteins, since residues involved in intein excision are not present at the C-terminal end of HalS8 and peptides removed from the middle of such excisions are nonfunctional. It is impossible to say if the excision sites are characteristic of any particular protease, since there is a lack of information on halobacterial proteases. It is possible that HalS8 may be processed and externalized by a hitherto unknown dedicated protease and transporter system analogous to that used by many small bacteriocins. Alternatively, it may be released by the lysis of a subpopulation of producer cells, as is the case for some colicins. It is tempting to speculate that the 230-amino-acid protein and the 45-amino-acid peptide play roles in halocin induction, processing, externalization or immunity, but antibody studies must be done first to eliminate the possibility that these two peptides are simply degraded. BLAST searches uncover no matches that could shed light on any possible functions for these two proteins [47].

Halocin HalR1, a second microhalocin

Halocin HalR1 is produced by the partially characterized haloarchaeon, *Hbt. salinarum* GN101, originally isolated from Guererro Negro, Mexico [9]. Like most halocins, HalR1 activity is first detected in culture supernatants at the transition to stationary phase [56] (O'Connor E and R Shand, unpublished). Onset of halocin activity is closely tied to culture density, and is independent of growth rate, oxygen starvation, amino acid starvation, nitrate limitation and phosphate limitation [56] (O'Connor E and R Shand, unpublished). Unlike HalH6, HalR1 does not appear to be archaeolytic, since it causes no change in the optical density or cell morphology of stationary phase *Hbt. salinarum*, nor can it form zones of inhibition on fully grown lawns [48]. HalR1 has a dose-dependent, archaeostatic effect on growing *Hbt. salinarum* [48].

Like HalS8, HalR1 activity is unaffected by desalting and is resistant to acids, bases and organic solvents [48,56] (O'Connor E

and R Shand, unpublished). HalR1 activity is resistant to DNase and RNase, and specific proteases such as papain, trypsin or thermolysin, but is sensitive to general proteases such as proteinase K, pronase P and elastase [48]. HalR1 activity has been stable for 7 years at 4°C (O'Connor E and R Shand, unpublished). Although not as thermostabile as HalS8, HalR1 retains 100% of its activity after incubation at 60°C for 24 h, but loses all of its activity after 5 min at 93°C (O'Connor E and R Shand, unpublished). Rdest and Sturm [48] isolated HalR1 activity as both large and small molecular weight forms and have suggested the possibility of a "carrier protein" for the larger form of this halocin.

HalR1 has been purified using a protocol similar to that used for HalS8, and has been sequenced by Edman degradation (see below) (O'Connor E and R Shand, unpublished). The sequence confirms that HalR1 is a microhalocin (38 amino acids), and even more intriguing, HalR1 is 63% identical and 71% similar to HalS8 (identical residues in capitals) [47] (O'Connor E and R Shand, unpublished):

HalR1 : lqsNINiNTAAaVILiFNQVqvgALCaPTpVsGGgPpP
HalS8 : sdcNINsNTAAdVILcFNQVgscALCsPTlV−GG−PvP

The small differences between these two peptides are compelling, since they must be responsible for the differences in the activity spectra and heat resistance. For example, HalS8 has four cysteines, while HalR1 only has one. Given that HalS8 and HalR1 are not cationic like typical bacteriocins and eucaryocins, it will be interesting to discover their mechanisms of action.

Sulfolobicins

The archaeocins produced by *Sulfolobus* are entirely different from halocins, since their activity is predominantly associated with the cells and not the supernatant [46]. Prangishvili *et al.* were the first to isolate and characterize these proteinaceous toxins, which they called "sulfolobicins" in keeping with bacteriocin nomenclature. Forty-one strains of *Sulfolobus* collected from solfataric fields in Iceland were characterized and all were members of one species, which has been provisionally named "*S. islandicus*". [46].

Screening for sulfolobicin activity involves spotting samples of exponentially growing "*S. islandicus*" cells on lawns of a sensitive strain of *S. solfataricus* P1. After 48 h at 80°C, all of the 41 isolates produce nearly clear zones with sharp borders. The size of the zone of inhibition is not affected by time, although it is affected by the concentration of sensitive cells in the lawn: when the concentration is decreased four-fold, the size of the zone increases three-fold. The reverse effect is seen when the concentration is increased. Tests for infectivity exclude the possibility that sulfolobicin activity is due to the presence of a virus [46].

To date, the spectrum of sulfolobicin activity appears to be restricted to other members of the sulfolobales. When spotted on lawns, all 41 producer strains inhibit *S. solfataricus* P1, *S. shibatae* B12 and six nonproducing strains of "*S. islandicus*". They do not inhibit each other or *S. acidocaldarius* DSM639, nor does purified sulfolobicin from strain HEN2/2 inhibit *Hbt. salinarum* R1 or *E. coli*. The fact that all 41 producer strains share cross-immunity and the same inhibition spectrum suggests that their sulfolobicins also share mode of action [46].

Unlike halocins, sulfolobicins are not secreted into the culture medium. To visualize activity by spotting directly on lawns, culture supernatants have to be concentrated 100-fold, either by precipitation or centrifugation, before extracellular sulfolobicin activity can be detected. Maximal levels of extracellular activity are obtained when cultures reach stationary phase. Analysis of sulfolobicin activity in a 500-ml culture reveals that 30 times more activity can be purified from cell pellets than from culture supernatant. Release of activity into the medium from exponentially growing cells is not induced by UV, cold shock (80−25°C), pH shock (pH 3−7) or by the presence of sensitive cells [46].

Extracellular activity is associated with spherical particles 90–180 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 reveal an inner core with a surrounding layer having a periodicity of 22 nm — the same as the lattice constant of the *Sulfolobus* S-layer [46].

Sulfolobicin is purified by 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 30−40 kDa on size exclusion chromatography in contrast to 20 kDa on SDS-PAGE. These data suggest that this archaeocin may aggregate [46].

Purified sulfolobicin has the same spectrum of activity as the producer strains and activity remains stable after 6 months at 4°C or 5 days at 85°C. Enzymatic treatment with α-amylase, α- and β-glucosidases, lipase, phospholipase C and lipoprotein lipase has no effect on activity. However, treatment with pronase E, proteinase K and trypsin completely destroys activity, indicating that activity is associated with a proteinaceous component [46].

Sulfolobicin concentrations of up to 3.5 AU/ml kill sensitive cells at the rate of about 1 log for every AU/ml. Thereafter, killing is not as effective, requiring a concentration of 11 AU/ml to increase mortality an additional half log. The LD_{50} of sulfolobicin for an *S. solfataricus* culture with an optical density of 0.25 at 600 nm is 100 AU/ml. After 20 min under these conditions, there is no change in optical density of the culture, but colony-forming units decrease by 50%. This was taken as evidence that the mode of sulfolobicin action is archaeocidal and not archaeolytic [46].

Sulfolobicins exhibit some classical bacteriocin characteristics in that they are proteinaceous and are directed against strains that are closely related to the producer. Although some of the producer strains contained conjugative plasmids, neither sulfolobicin production nor immunity can be transferred to nonproducer strains. This suggests that the genes for these traits may be chromosomal. While this study 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 is also seen with cell-bound bacteriocins [46].

Activity spectra

As mentioned above, activity spectra of various bacteriocins and eucaryocins vary widely. The same is true for the haloarchaea when tested against other haloarchaea [35,64]. However, Hazeltine *et al.* have shown that three microhalocins, HalR1, HalS8 and HalA4, are active against three species of *Sulfolobus*, with HalR1 and HalS8 cytostatic and HalA4 cytocidal (halocin A4 is produced from a

haloarchaeon isolated from a saltern in Tunisia and is currently being characterized) [17]. This is an important finding as *Sulfolobus* is a hyperthermophilic member of the kingdom Crenarchaeota, while the haloarchaea are members of a different archaeal kingdom, the Euryarchaeota. In addition, *Sulfolobus* mutants resistant to HalA4 were isolated and found to be stable. These data beg the question of whether *Sulfolobus* and the haloarchaea share a common archaeal-specific target. Furthermore, a preliminary experiment showed that HalR1 was active against the methanogen *Methanosarcina thermophila* (a euryarchaeote), while HalS8, HalA4 and HalH4 were not.

In addition to killing *Sulfolobus* and inhibiting most haloarchaea it contacts, HalA4 also inhibits two haloalkaliphiles that grow optimally at pH 9.5, *Natronobacterium gregoryi* and *Natrialba magadii*, but not *Natronomonas pharaonis* or *Natronococcus occultus* (Kamadulski A and R Shand, unpublished).

Nisin, a class I lantibiotic (see above) produced by *Lactococcus lactis*, is also active against the hyperthermophilic crenarchaeote *S. acidocaldarius* (D. Grogan, personal communication). Grogan and Clark were able to isolate stable nisin-resistant mutants as well. Exposure of wild type *S. acidocaldarius* to nisin resulted in release of 60% of the cellular ATP compared to only 20% from a nisin-resistant mutant (D. Grogan, personal communication).

Biotechnology applications

The two microhalocins, halocins S8 and R1, are unusual in structure since they are not cationic and apparently do not fold into amphipathic α-helices. Although both are very hydrophobic, they do not affect Gram-positive or Gram-negative bacteria, or the yeast *Saccharomyces cerevisiae* or *Candida albicans* (Lewis S and R Shand, unpublished). Bacterial and eucaryal peptide antibiotics that affect bacteria and lower eucaryotes are frequently cationic and form amphipathic α-helices or β-sheets, and work by binding to anionic membrane lipids and lipopolysaccharides as a prelude to membrane disruption. Microhalocins lack the biochemical and structural properties that peptide antibiotics employ to kill or inhibit bacteria and lower eucaryotes by membrane disruption. Given their neutrality and hydrophobicity, their mechanisms of action will almost certainly be novel.

The mechanism of action is known for halocin H6: disruption of the haloarchaeal Na^+/H^+ antiporter [36]. In mammals, Na^+/H^+ antiporter inhibitors protect the myocardium against ischemia and reperfusion injury [37]. This led to the isolation of a halocin H6 overproducer strain (*Hfx. mediterranei* Alicante SPH7, the halocin from which has been renamed halocin H7) and testing of this haloarchaeal Na^+/H^+ antiporter inhibitor in a dog model [1]. Halocin H7 treatment protected the myocardium against the deleterious effects of ischemia and reprefusion by decreasing infarct size and the number of ectopic beats. This finding has implications for reducing injury during organ transplantation.

There are few selectable markers in the domain Archaea, especially outside of the Halobacteriaceae, which severely limits the ability to perform genetic studies. The discovery of halocins that are active on organisms outside of the Halobacteriaceae offers the hope of archaeocin-resistant markers. In addition, the fact that HalH7 inactivates the same target in mammals as it does in halobacteria suggests the possibility that other archaeocins may have clinical applications. This should encourage the search for more of these peptides and their continued study.

To realize their full clinical potential, future studies on archaeocins must focus on their physical structures and their modes of action. This information will give clinicians the ability to predict which archaeocins will have desirable pharmaceutical effects. In addition, a variety of structure/function studies, such as peptide modeling, site-directed mutagenesis or side-chain alteration, may suggest which archaeocins have the greatest potential for combinatorial studies. Finally, it is equally important to understand the basic biology behind how the producer achieves immunity, the mechanism(s) involved in halocin secretion and processing, the signal(s) involved in induction, the factors and elements involved in gene regulation, and ribosome binding to leaderless transcripts.

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