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Halocins: are they involved in the competition between halobacteria in saltern ponds?

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Abstract Many representatives of the family *Halobacteriaceae* ("halobacteria") excrete halophilic bacteriocins (halocins) that inhibit the growth of other halobacteria. In spite of the fact that halocin production is widespread among the *Halobacteriaceae*, no information is available on their ecological significance. To test whether halocins may play a role in the interspecies competition between different types of halobacteria in saltern crystallizer ponds inhabited by dense communities of these red halophiles, we assayed for halocins active against a variety of halobacteria in salterns from different locations worldwide. Detection of halocin activity was based on the inhibition of growth of indicator organisms on agar plates, the decreased incorporation of radiolabeled substrates, and microscopic examinations. No halocin activity was detected in any of the brines examined, in spite of the fact that halocin production was demonstrated in cultures of most microorganisms isolated from these brines. Thus, the contribution of halocins in the competition between different halobacteria in hypersaline aquatic environments is probably negligible.

Key words Halocins · Halobacteria · Salterns

Introduction

Salt lakes and saltern ponds, at or close to NaCl saturation, worldwide are inhabited by dense communities of halobacteria (family *Halobacteriaceae*). Densities of 10^7–10^8 cells ml^{-1} and higher are common. Because of their high content of C-50 carotenoids (α-bacterioruberin and derivatives), they impart a reddish color to the brines (Oren 1993, 1994a; Tindall 1992).

Representatives of the family *Halobacteriaceae* require high NaCl concentrations for growth. The nutritional requirements of the known species are similar (possibly as a consequence of the use of similar enrichment and isolation conditions): they are commonly grown in rich media containing amino acids and yeast extract. Some have a limited ability to use sugars as well, and a few isolates, notably those belonging to the genera *Haloferax* and *Haloarcula*, can grow in defined media on a range of simple compounds (sugars, organic acids) as the single carbon and energy source. At the time of writing (July 1999), the family consisted of 14 genera with 35 validly described species, of which 10 are alkaliphiles and the rest are neutrophiles. The present classification is based mainly on properties such as 16S rRNA sequences, polar lipid composition, cell morphology, and a number of phenotypic properties (Kamekura 1998; Tindall 1992).

Although a variety of halobacteria can be isolated from hypersaline lakes, one or at most a small number of genera appear to dominate in the brines. Polar lipid analysis of natural communities showed the presence of a single glycolipid (probably identical to the sulfated diglycosyl diether lipid S-DGD-1 characteristic of the genera *Haloferax*, *Halobaculum*, and *Halococcus*). This condition was true for the Dead Sea at the time of a massive halobacterial bloom (Oren and Gurevich 1993) and for the saltern crystallizer ponds of Eilat, Israel (Oren 1994b; Oren et al. 1996). A similar picture was found for the salterns of Alicante, Spain, and Newark, CA, USA: S-DGD-1 was the dominant glycolipid present in the community, and other glycolipids, if detected at all, were present in minor amounts only (this study; Oren and Litchfield, unpublished results). Evidence is accumulating that the dominant halobacterium in saltern crystallizers has not yet been isolated. Analysis of 16S rDNA sequences amplified directly from the Alicante saltern ponds consistently yielded sequences that, although

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being very similar to each other, differed greatly from those of all genera recognized within the *Halobacteriaceae* (Benlloch et al. 1995; Rodríguez-Valera et al. 1999). The same phylotype was also detected as the dominant type in the Eilat crystallizers (Rodríguez-Valera et al. 1999).

Little is known about the factors that allow certain populations of halobacteria to reach high densities to the exclusion of others. One factor that may give halobacteria a competitive advantage is the action of halophilic bacteriocins (halocins). Bacteriocins are proteins capable of inhibiting organisms closely related to the producer. The formation of bacteriocins by a member of the *Halobacteriaceae*, *Haloferax mediterranei*, was first described in 1982 (Rodriguez-Valera et al. 1982, 1983). It has been documented since that halocin production is an almost universal feature among the halobacteria (Meseguer et al. 1986; Shand et al. 1999; Torreblanca et al. 1994). A wealth of information has accumulated on halocins, but we lack direct evidence that they are produced in the natural environment by halobacteria in sufficient quantities to inhibit potential competitors. Our understanding of the ecological implications of halocin production has been summarized by Oren (1994a): "Halocins provide the species that produce them with a means to avoid competition by other species that have the same environmental requirements. However, all our knowledge on the halocins was gathered in laboratory cultures, and no information is available as yet on the importance of bacteriocins during competition between different members of the *Halobacteriaceae* under field conditions."

In the present study we attempted to assess the presence and potential importance of halocins in the ecology of halobacteria in aquatic hypersaline environments.

Materials and methods

Environmental samples and sample preparation

Samples were collected from crystallizer ponds of the salterns of the Israel Salt Company at Eilat (Red Sea coast) (5 July 1997, 11 August 1997, 2 February 1998, 17 November 1998, 10 January 1999, and 30 June 1999), and at Atlit (Mediterranean coast) (14 August 1998). Additional samples were obtained from the crystallizers of the Cargill Solar Salt Company, Newark, CA, USA (15 February 1997 and 15 January 1998) and from the Santa Pola salterns near Alicante, Spain (11 September 1997). The bacterial community density in the samples was determined microscopically in a Petroff–Hauser counter if necessary after concentration by centrifugation (15 min, 12 000 × g). *Dunaliella* cells were counted by filtering samples through Millipore filters (5 μm pore size) and counting cells on the filter under a 16× objective.

Samples used for bacteriocin activity assays were centrifuged (20 min, 15 000 × *g*) within 2 h of sampling to remove bacteria, and were kept at room temperature until further processing (usually within 1–2 days). In some of the experiments, macromolecules in the brines were concentrated by ultrafiltration using the Millipore Minitan II ultrafiltration system. Brine samples (1–2.5 l) were first freed of bacteria by filtration through Durapore GVLP filter plates (0.2 μm pore size), followed by 10- to 53.5-fold concentration of the macromolecular fraction on regenerated cellulose filters (Millipore PLAC or PLCC with nominal molecular weight cutoff values of 1000 or 5000, respectively). The protein content of the concentrates was determined by adding cold 100% trichloroacetic acid to ice-cold samples to a final concentration of 10% and collecting precipitated protein by centrifugation, followed by quantification using the Lowry procedure (Lowry et al. 1951).

Halobacterial strains and culture conditions

Halobacteria were grown in 100 ml of medium in 250-ml Erlenmeyer flasks in a rotatory shaker at 35°C. The following media were employed (concentrations in $g l^{-1}$): (A) NaCl, 250; KCl, 5; $MgCl_2 \cdot 6H_2O$, 5; NH_4Cl, 5, and yeast extract, 10; (B) NaCl, 206; $MgSO_4 \cdot 7H_2O$, 36; KCl, 0.37; $CaCl_2 \cdot 2H_2O$, 0.5; $MnCl_2$, 0.013, and yeast extract, 5; (C) NaCl, 175; $MgCl_2 \cdot 6H_2O$, 20; K_2SO_4, 5, $CaCl_2 \cdot 2H_2O$, 0.1, and yeast extract, 5; (D) NaCl, 158; $MgCl_2 \cdot 6H_2O$, 13; $MgSO_4 \cdot 7H_2O$, 20; KCl, 4; $CaCl_2 \cdot 2H_2O$, 1; NaBr, 0.5; $NaHCO_3$, 0.2; yeast extract, 5; tryptone, 8, and glucose, 1; (E) NaCl, 200; KCl, 1; $MgSO_4 \cdot 7H_2O$, 20; $FeSO_4 \cdot 7H_2O$, 0.02; trisodium citrate, 3; casamino acids, 7.5, and yeast extract, 0.5; (F) NaCl, 125; $MgCl_2 \cdot 6H_2O$, 160; K_2SO_4, 5; $CaCl_2 \cdot 2H_2O$, 0.1; yeast extract, 1; casamino acids, 1, and starch, 2. All media were adjusted to pH 7.0 with NaOH. For solid media, agar was added at $20 g l^{-1}$.

The following reference cultures of halobacteria were used in halocin assays, the letters in brackets indicating the growth medium employed: *Halobacterium salinarum* NRC 817 (A), *Halobacterium salinarum* (*halobium*) R1 (A), *Halobacterium salinarum* strain 5 (Simon 1980) (A), *Haloarcula marismortui* ATCC 43049^T (B), *Haloarcula vallismortis* ATCC 29715^T (B), *Haloferax mediterranei* ATCC 33500^T (C or D), *Haloferax volcanii* ATCC 29605^T (C), *Haloferax gibbonsii* ATCC 33959^T (C), *Haloferax denitrificans* ATCC 35960^T (C), *Halorubrum saccharovorum* ATCC 29252^T (E), *Halorubrum sodomense* ATCC 33755^T (F), and *Halobaculum gomorrense* DSM 9297^T (F).

To assess the efficiency of the halocin concentration procedure by ultrafiltration used for environmental samples (see above), *Haloferax mediterranei* ATCC 33500 was grown in 1 l portions of medium D in 3-l Erlenmeyer flasks at 35°C in a rotatory shaker. At the beginning of the stationary growth phase, cells were removed by centrifugation (15 min, 8000 × *g*), whereafter the supernatant (1.75 l) was filtered through Durapore GVLP filter plates (0.2 μm pore size), followed by 28.5-fold concentration of the macromolecular fraction on Millipore PLAC filters (nominal molecular weight cutoff value, 1000), in a treatment similar to that to which the environmental samples were subjected (see above).

An additional 41 strains of halobacteria were isolated from the Eilat salterns, 20 by direct plating of 10-μl portions on plates (media A, C [with NaCl concentration reduced to 125 g l^{-1} and $MgCl_2 \cdot 6H_2O$ increased to 50 g l^{-1}], and E), and 21 from enrichment cultures (100-ml portions of brine, diluted with distilled water to 80% or 90% of the original salinity, and enriched with yeast extract (2 g l^{-1}) or with a combination of glycerol (2 g l^{-1}) and yeast extract (1 g l^{-1}).

Environmental samples and isolated cultures were characterized by polar lipid analysis, using thin layer chromatography on silica gel plates as described by Oren and Gurevich (1993) and Oren et al. (1996). Lipid spots were visualized with $CeSO_4$-H_2SO_4, orcinol-$FeCl_3$ stain (Sigma), α-naphthol-H_2SO_4 (for detection of glycolipids), and molybdate spray (for identification of phospholipids) (Oren et al. 1996).

Assays of halocin activity

Halocin assays were based on the formation of clear zones on double layer agar plates (Meseguer et al. 1986; Torreblanca et al. 1994). Plates with 15 ml of suitable medium solidified with 2% agar were overlaid with 7.5 ml of molten (50°C) agar medium (1% agar) of the same composition, to which approximately 10^7 cells of the indicator bacterium (100 μl culture) were added. Samples to be tested for halocin activity against the indicator bacterium (halobacterial colonies, liquid cultures, or filtrates) were directly applied on the agar (10 μl for liquid samples). In some experiments, 30-μl portions of samples were added to wells (6 mm diameter) cut into the upper agar layer. Plates were incubated for 2 or more days at 35°C until confluent growth of the indicator bacteria was achieved. Culture supernatants of *Haloferax mediterranei* were used as positive controls, using *Halobacterium salinarum* NRC 817 as an indicator strain. If necessary, twofold dilution series were made in sterile growth medium. The highest dilution producing visible inhibition was considered as containing one arbitrary unit (AU).

Measurement of uptake of radiolabeled substrates

Saltern brines were freed of bacteria by centrifugation (10 min, 12000 × *g*), followed by filtration through 0.2-μm-pore-diameter Millipore filters. Cells from 4-ml portions of early exponential phase cultures of *Halobacterium salinarum* NRC 817 were collected by centrifugation (10 min, 12000 × *g*) and suspended in 10 ml of the brines tested. Control experiments included use of heat-treated brines (boiling for 10 min or autoclaving for 25 min to inactivate those halocins that are heat sensitive), and addition of 10 ml of culture supernatant of a late-exponential culture of *Haloferax mediterranei* as a positive control. Tubes were preincubated at 37°C for periods varying from 4 to 24 h to allow halocins to interact with the target cells. Portions of 10 μl of [U-^{14}C]glycerol (Amersham; 50 μCi ml^{-1}, 153 mCi $mmol^{-1}$) or a ^{14}C-labeled amino acid mixture (Amersham; 50 μCi ml^{-1}, 57 mCi mmol C^{-1}) were then added, and incubation at 37°C was continued. Samples (2 ml) were withdrawn at 10- to 20-min intervals, and filtered through glass fiber filters (Whatman GF/C). The filters were washed with cold 10% trichloroacetic acid, and radioactivity incorporated by the cells was quantified in a Beckman LS 1801/2800 scintillation counter with 5 ml Zinsser Quicksafe scintillation cocktail.

Results

We examined a number of crystallizer brine samples, collected from different geographic locations and in different seasons (Table 1). With the exception of the samples from the Cargill plant collected in February 1997 following a period of heavy rains, a crust of precipitated NaCl was found on the bottom of all ponds sampled. Total microscopic bacterial counts varied from 8.4×10^6 ml^{-1} (Eilat in winter) to 7.1–7.2 × 10^8 ml^{-1} (Newark and Santa Pola). Polar lipid analyses demonstrated that the prokaryote community in all samples was dominated by halobacteria. The lipid

Table 1. Brine samples examined for the presence of halocins

Location	Sampling date	Brine density (g ml^{-1})	Bacterial counts (cells ml^{-1})	*Dunaliella* counts (cells ml^{-1})
Eilat, Israel	5 July 1997	1.235	3.5×10^7	620
	11 August 1997	1.235	1.4×10^7	1000
	2 February 1998	1.24	1.5×10^7	400
	17 November 1998	1.24	1×10^7	460
	10 January 1999	1.24	8.4×10^6	480
	30 June 1999	1.244	1.8×10^7	2700
Atlit, Israel	14 August 1998	1.244	1.38×10^8	ND
Newark, California	15 February 1997			
Pond 26		1.173	7.1×10^8	12500
Pond 10A		1.175	1.5×10^8	9000
	24 July 1998			
Pond 10A		1.21	1×10^8	930
Pond 11		1.22	1.5×10^8	4600
Santa Pola, Spain	11 September 1997	1.235	7.2×10^8	ND

pattern obtained was similar to that reported earlier for the Eilat samples (Oren 1994b; Oren et al. 1996), major lipid spots being identified as the phytanyl diether derivatives of phosphatidylglycerol, the methyl ester of phosphatidylglycerophosphate, phosphatidylglycerosulfate, and a single glycolipid, chromatographically identical to the S-DGD-1. Additional minor glycolipid spots were detected in the Newark samples. As judged from the lipid patterns, the contribution of representatives of the domain Bacteria to the prokaryote community in the ponds was negligible in all cases.

To test for the presence of halocin activity in the saltern brines inhabited by dense communities of halobacteria, we removed cells by centrifugation, followed by filtration of the supernatant through 0.2-μm-pore-size Millipore filters. The cell-free brines were applied to double-layer agar plates with all the 12 indicator organisms used, adding the samples both as drops on the developing lawn of the indicator organisms and in wells cut into the upper agar layer. No growth inhibition zones of any of the indicator organisms were obtained with any of the brine samples examined. Supernatants of late exponential growth phase cultures of *Haloferax mediterranei* served as a positive control in these experiments.

To test whether halocins may have been present in the samples below the detection level of the double-layer agar plate method, we concentrated the macromolecular fraction in two Eilat brine samples by ultrafiltration. The 17 November 1998 sample was concentrated 10 fold using a filter with a nominal cutoff size of 5000 molecular weight after prefiltration through a 0.2-μm filter to remove bacteria, algae, and other particles. The concentrate did not show halocin activity. The samples of 17 November 1998, 10 January 1999, and 30 June 1999 were concentrated using a 1000 molecular weight cutoff filter to avoid loss of small halocins ("microcins"), if present. Also these concentrates (24.3-, 26.7-, and 53.5-fold concentration, containing 9.6, 10.4, and 6.0 $\mu g\,ml^{-1}$ dissolved protein, respectively) failed to inhibit any of the indicator organisms used on the agar plates, both when applied as 10-μl droplets on the agar and as 30-μl portions in wells cut in the agar. To assess the efficiency of the filtration procedure used, culture supernatant of a late exponential – early stationary phase culture of *Haloferax mediterranei* (1.6×10^9 cells ml^{-1}) was treated in an identical way, using *Halobacterium salinarum* NRC 817 as indicator organism. Culture supernatant samples before filtration and concentration contained 2–4 AU of halocin activity by the drop method; following 28.5-fold concentration using a 1000 molecular weight cutoff filter, 32–64 AU were measured in samples assayed after 1–2 weeks storage at room temperature, 4°C, −20°C, and −80°C, being about half the theoretically expected activity (see also Discussion).

Additional approaches were used to assess the extent of antagonistic interactions of macromolecules present in the brines with a variety of culture collection strains of halobacteria. Brine supernatants and concentrates obtained by ultrafiltration (see above) were added to cell suspensions in a ratio of 9:1 by volume (final cell density, about 10^8 cells ml^{-1}) and incubated at 35°C for periods up to 18 h. Microscopic examination showed that cells maintained their native morphology and motile strains retained their motility. Viability was maintained throughout, as shown by streaking 10-μl samples on agar plates of suitable media. No inhibition of the incorporation of ^{14}C-labeled glycerol or amino acids by *Halobacterium salinarum* by Eilat saltern brine supernatants was observed; in control experiments with *Haloferax mediterranei* culture supernatant, up to 50% inhibition of the incorporation was obtained.

Forty-one strains of halobacteria were isolated in the course of the study from the Eilat salterns. On the basis of polar lipid analysis 12 isolates could be assigned to the genus *Halobacterium*, 9 to *Haloarcula*, and 5 to *Halorubrum*; no polar lipid tests were performed on the remaining strains. Of the 41 isolates, 29 showed halocin activity against at least 1 of the indicator strains used, with 13 strains inhibiting 5 or more of the indicator strains (Table 2). Especially abundant were strains inhibiting *Halobacterium salinarum* strains (26), *Haloarcula vallismortis* (19), *Haloarcula marismortui* (16), *Halobaculum gomorrense* (16), and *Halorubrum sodomense* (15). None of these isolates was antagonistic to *Haloferax mediterranei*, *Haloferax volcanii*, *Haloferax denitrificans*, or *Halorubrum saccharovorum*.

Discussion

Halobacteria inhabit hypersaline environments in which only very few other organisms are able to live. They therefore compete almost exclusively with each other. The production of bacteriocin-like substances could thus provide a mechanism by which species avoid competition with other species that have the same environmental requirements. Halocin production was found to be a common feature of halobacteria. Many different halocins are produced by this heterogeneous group of microorganisms. Some strains inhibit many test organisms, and others have a narrow specificity (Torreblanca et al. 1994).

Comparative studies with large numbers of isolates have shown that there should be at least 15 different types of halocins with different activity spectra. Four halocins have been investigated in great depth: halocin H4 of *Haloferax mediterranei*, halocin H6 of *Haloferax gibbonsii*, halocin Hal R1 of *Halobacterium* sp. GN 101, and halocin S8 of a yet uncharacterized rod-shaped halobacterium. Halocin H4 is a 28-kDa heat-sensitive and salt-dependent protein. It interacts with the membrane of the target cells, where it causes permeability changes that result in an ionic imbalance, leading to death and cell lysis (Meseguer and Rodriguez-Valera 1985; Meseguer et al. 1991; Rodriguez-Valera et al. 1982). Sensitive cells become swollen and spherical (Meseguer and Rodriguez-Valera 1986). Halocin H6 has a size of 32 kDa, is heat resistant, and is non-salt dependent (Torreblanca et al. 1989). It specifically inhibits the Na^+/H^+ antiporter of sensitive cells, thus targeting the central device used by the halobacteria to adapt to highly

Table 2. Halocin activity of 41 halobacterial strains isolated from the Eilat crystallizer ponds

Strain no.	Glycolipids	Inhibition of indicator strain										
		A	B	C	D	E	F	G	H	I	J	K
1–4	S-TGD, S-TeGD	+	+	+	+	+	+	+	−	−	−	−
5–7	S-TGD, S-TeGD	+	+	+	+	+	+	−	−	−	−	−
8–9	TGD-2	+	+	+	+	+	+	−	−	−	−	−
10	ND	+	+	−	+	+	+	+	−	−	−	−
11	S-TGD, S-TeGD	+	+	+	−	+	+	−	−	−	−	−
12	ND	+	+	−	−	+	+	+	−	−	−	−
13–15	S-TGD, S-TeGD	+	+	+	+	−	−	−	−	−	−	−
16	S-DGD	+	+	+	+	−	−	−	−	−	−	−
17	ND	+	+	+	−	−	+	−	−	−	−	−
18	ND	+	+	+	−	−	−	−	−	−	−	−
19	ND	+	+	−	−	+	−	−	−	−	−	−
20	TGD2	−	+	−	−	+	+	−	−	−	−	−
21	ND	−	−	+	+	+	−	−	−	−	−	−
22–26	ND	+	+	−	−	−	−	−	−	−	−	−
27	ND	+	−	+	−	−	−	−	−	−	−	−
28	ND	−	−	+	+	−	−	−	−	−	−	−
29	TGD-2	−	−	−	−	+	−	−	−	−	−	−
30–35	TGD-2	−	−	−	−	−	−	−	−	−	−	−
36–39	S-DGD[a]	−	−	−	−	−	−	−	−	−	−	−
40	S-TGD, S-TeGD(?)	−	−	−	−	−	−	−	−	−	−	−
41	ND	−	−	−	−	−	−	−	−	−	−	−

S-TGD and S-TeGD, the sulfated triglycosyl and tetraglycosyl diether lipids characteristic of the genus *Halobacterium*; TGD-1, the triglycosyl diether lipid characteristic of the genus *Haloarcula*; S-DGD, a sulfated diglycosyl diglyceride that was not further identified

Indicator strains: A, *Halobacterium salinarum* R1; B, *Halobacterium salinarum* strain 5; C, *Haloarcula vallismortis*; D, *Haloarcula marismortui*; E, *Halobaculum gomorrense*; F, *Halorubrum sodomense*; G, *Haloferax gibbonsii*; H, *Haloferax mediterranei*; I, *Haloferax denitrificans*; J, *Haloferax volcanii*; K, *Halorubrum saccharovorum*.

[a] PGS present

saline environments (Meseguer et al. 1995). Halocin Hal R1 is small (6.2 kDa; to be classified as a "microcin"), thermostable, non-salt dependent, and has a bacteriostatic effect on a wide variety of halobacteria (Rdest and Sturm 1987). Halocin S8 is another microcin, with an apparent molecular mass of 2.5 kDa, and is also thermostable and not dependent on salt (Shand et al. 1999). A general overview of the properties of these halocins was given by Shand et al. (1999). All four halocins mentioned above are excreted when cultures of the producing strains enter the stationary growth phase (Shand et al. 1999). In *Haloferax mediterranei*, the transcript for halocin H4 is present at a low level during exponential growth but increases drastically at the end of the exponential growth phase when resources become exhausted (Cheung et al. 1997). Activities of halocins H4, H6, and S8 decrease during the stationary phase, but HalR1 maintains a high activity for prolonged times (Shand et al. 1999).

We were unable to demonstrate halocin activity in saltern brines from different geographic locations, even after concentration by ultrafiltration, using a variety of sensitive test strains. The use of filters with a nominal molecular weight cutoff value of 1000 would ensure also the concentration of microcins similar to halocin Hal R1 and S8, if present. The 30 June 1999 sample (1.8×10^7 cells ml^{-1}), after 53.5-fold concentration, would have yielded a concentrate corresponding to a cell density of $10^9 ml^{-1}$, approximately equivalent to that of a fully grown cultures of halobacteria in rich medium. If halocins were present they should have been detectable, even if half the activity was lost in the course of the filtration procedure. 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. However, certain possible problems should be taken into account. Recovery of halocin H6 during ultrafiltration was reported to be less than ideal, probably due to nonspecific adsorption of the protein to the filters (Torreblanca et al. 1989). Also, in our case it is possible that the concentrate contained less activity than expected because of adsorption of halocin to the filters. A control experiment with *Haloferax mediterranei* showed a loss of approximately half the activity in the course of the filtration procedure. Classic methods of protein concentration such as ammonium sulfate precipitation are ineffective with the salt-dependent proteins of the halobacteria, and more efficient concentration protocols for the halocins that are salt independent have yet to be developed. Another possible cause of the apparent lack of halocin activity in the samples may be the activity of halophilic proteases excreted by some halobacteria (e.g., Kamekura and Seno 1993). Such proteases could have destroyed halocins that may have been present at the time of sampling during the time (up to 1–2 days) that had passed during transportation of the samples to the laboratory and during sample processing (e.g., ultrafiltration).

Although the excretion of bacteriocins can be expected to provide a competitive advantage to a bacterium that must compete with sensitive organisms which, being closely related, use the resources of the environment in much the same way, we still know very little of the role that bacteriocins play in nature in determining the outcome of the competition between bacteria (see review articles by Konisky 1982; Reeves 1965, 1972; Tagg et al. 1976). In homogenous environments bacteriocin formation has little competitive value because the killing of sensitive cells by the bacteriocin augments the amount of resource available to the bacteriocin-producing bacteria to an extent identical to that experienced by the surviving sensitive bacteria (Chao and Levin 1981; Hoyt and Sizemore 1982). In structured, heterogeneous environments, however, bacteriocin-producing bacteria may have a pronounced selective advantage (Chao and Levin 1981; Durrett and Levin 1997). For example, they may play a role in the territorial interactions between different *Myxococcus* species (Smith and Dworkin 1994).

The high community densities of halobacteria would make the saltern crystallizers an ideal study site for research on the importance of bacteriocins in the natural environment. The present study has failed to show halocin activity in halobacterial communities in salterns. Therefore, the question of the ecological importance of halocins remains unanswered. In view of the dependence of halocin excretion on the physiological state of the cells (Cheung et al. 1997; Meseguer and Rodriguez-Valera 1985; Shand et al. 1999), it may be worthwhile to perform assays similar to those described here in different seasons and at different stages during the buildup of the halobacterial community after a crystallizer pond is filled with new brine.

It remains unknown whether the yet uncultured phylotype that dominates the halobacterial communities in salterns in Spain and in Israel (Benlloch et al. 1995; Rodríguez-Valera et al. 1999) produces halocins, thereby exterminating its competitors by chemical warfare. The field studies reported here do not provide strong evidence, but the question can only be answered definitively after the organism has been obtained in culture. Bacteriophages also appear to be of minor importance in the control of halobacterial abundance and growth rate in saltern ponds (Guixa-Boixareu et al. 1996).

A possible role for halocins in the interspecies competition between different types of halobacteria can be envisaged on solid substrates such as salted fish and hides. Here the halophiles develop as red colonies, and the localized dense cell masses may well excrete halocins that prevent the development of colonies of competitor species in the neighborhood, similar to that which is observed on agar plates. The presence and role of halocins in such environments deserve an in-depth study.

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