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Purification and biological characterization of halocin C8, a novel peptide antibiotic from *Halobacterium* strain AS7092

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Abstract Halocins are bacteriocin-like proteins or peptides produced by many species of the family *Halobacteriaceae*. Halocin C8, excreted by the *Halobacterium* strain AS7092, is a single 6.3-kDa polypeptide with an isoelectric point of 4.4, which is sensitive to proteinase K but not to trypsin. Halocin C8 is quite stable, as it can be desalted, boiled, frozen, subjected to organic solvents, and stored in culture supernatant at 4°C or in dH_2O at −20°C for more than 1 year without losing activity. The purification of this halocin was achieved by combination of tangential flow filtration (TFF), Sephadex G50 and DEAE-sepharose chromatography. The N-terminal amino acid sequence was also determined by Edman degradation. Halocin C8 appeared to have a very wide activity spectrum, including most haloarchaea and even some haloalkaliphilic rods. When a sensitive strain of *Halorubrum saccharovorum* was exposed to halocin C8, the treated cells swelled at the initial stage, the cell wall appeared to be nicked and the cytoplasm was then extruded out, and the whole cell was eventually completely lysed. These results indicate that halocin C8 is a novel microhalocin and its primary target might be located in the cell wall of the sensitive cells.

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Introduction

Halocins are protein or peptide antibiotics produced by several extremely halophilic members of the domain *Archaea* (Rodriguez-Valera et al. 1982; Meseguer and Rodriguez-Valera 1986), one of the three primary domains of cellular organisms (Woese et al. 1990). To coincide with bacteriocins produced by members of the domain *Bacteria*, and eucaryocins produced by members of the domain *Eucarya*, the proteinaceous antibiotics produced by *Archaea* are termed as archaeocins (O'Connor and Shand 2002). Until recently, archaeocins were still limited to halocins from the extremely halophilic archaea, when a sulfolobicin was discovered from the hyperthermophilic crenarchaeal genus *Sulfolobus* (Prangishvili et al. 2000).

Halocin production is recognized as a nearly universal feature of haloarchaeal rods (Torreblanca et al. 1994). However, in contrast to their ubiquity, only a handful of halocins so far have been described in detail. The purified halocins include halocin H1 (Platas et al. 2002), H4 (Meseguer and Rodriguez-Valera 1985; Cheung et al. 1997), H6 (Torreblanca et al. 1989), S8 (Price and Shand 2000) and R1 (Rdest and Strum 1987; O'Connor and Shand 2002), while only two genes for halocin H4 (*halH4*) and S8 (*halS8*) have been cloned (Cheung et al. 1997; Price and Shand 2000). Most of these halocins are initially produced in the culture supernatants at the beginning of the transition into stationary phase, except for halocin H1, whose activity is first detectable during mid-exponential phase (O'Connor and Shand 2002; Platas et al. 1996). Thus, halocins are usually chosen as models to study phase-specific gene expression in the haloarchaea (Cheung et al. 1997; Price and Shand 2000). Halocins are diverse in size, ranging

from proteins as large as 35 kDa (e.g., H4) to peptides as small as 3.6 kDa (e.g., S8). The peptide halocins, also named as microhalocins, are usually quite robust, as most of them are resistant to acid, base, and organic solvent, and their activities are usually unaffected by desalting or boiling (Shand et al. 1999; Price and Shand 2000; and this work). These properties suggest that this kind of halocin may be also a potential model for protein structure/function studies.

Like bacteriocins in domain *Bacteria* or eucaryocins in domain *Eucarya*, the activity spectra of halocins vary widely when tested with other haloarchaea, some are broad (e.g., H1) and others are narrow (e.g., H4, H6), although the activities of halocins antagonizing bacteria have not yet been reported. Recently, it has been found that a few microhalocins (e.g., R1, S8, and A4) are also active against some species of *Sulfolobus* or *Methanosarcina*, the members of other two distinct archaeal groups, hyperthermophiles and methanogens (Haseltine et al. 2001; O'Connor and Shand 2002), which suggests that these different archaeal kingdoms may share a common archaeal-specific target. However, the primary targets of most halocins are still to be investigated. To date, only in one case (halocin H6) has the mechanism been revealed, where halocin H6 kills sensitive cells by specific inhibition of the halobacterial Na^+/H^+ antiporter (Meseguer et al. 1995). Since halocin activity spectra have been seen to vary so far, the primary target of each kind of halocin may be different.

To further investigate the diversity and mechanisms of halocins, and their potential in biotechnology applications, many more halocins still need to be purified and characterized. Halocin C8, produced by the halophilic archaeon *Halobacterium* strain AS7092, is a novel microhalocin. It is quite stable and was found to have a very broad inhibitory spectrum among different haloarchaea. In this paper, we describe the isolation, purification, N-terminal amino acid sequencing, and biological characterization of this halocin.

Materials and methods

Haloarchaeal and bacterial strains, media, and growth conditions

The producer of halocin C8 is *Halobacterium* strain AS7092, a partially characterized haloarchaeon isolated from the Great Chaidan Salt Lake in Qinghai province in the People's Republic of China. The other haloarchaeal strains used for the antagonism studies were: *Haloarcula hispanica* ATCC33960, *Har*. sp. MQ6; *Halorubrum saccharovorum* ATCC29252, *Hrr*. *trapanicum* JCM9743; *Halobacterium salinarum* ATCC33170, *Hbt*. *salinarum* DSM670, *Hbt*. sp. QD3; *Halococcus morrhuae* DSM1309, *Hcc*. *turkmenicus* JCM9101; *Haloferax denitrificans* ATCC35960, *Hfx*. *gibbonsii* ATCC33959, *Hfx*. *mediterranei* ATCC33500, *Hfx*. *volcanii* ATCC29605, *Hfx*. sp. JCM9276; *Natrinema versiforme* JCM10478, *Nnm*. sp. D20; *Natronobacterium gregoryi* NCMB2189; *Natrialba magadii* NCMB2190; *Natronomonas pharaonis* DSM2160; *Natrononococcus occultus* JCM8859; and *Natronorubrum tibetense* GA33. The bacterial strains used for the inhibitory tests were: *Staphylococcus aureus* CMCC(B)26001, *Pseudomonas aeruginosa* JCM5962, *Bacillus subtilis* CMCC(B)63003, *Bacillus cereus* CMCC(B)63303, and *Sarcina lutea* CMCC(B)28001. All these strains were kindly provided by the Type Culture Collection of the Chinese Academy of Sciences (Beijing, P.R. China).

Halobacterium and *Halococcus* strains were grown in AS-169 medium containing the following components per liter: 7.5 g Bacto casamino acids (Difco), 10.0 g Bacto yeast extract (Difco), 250 g NaCl, 20 g $MgSO_4{\cdot}7H_2O$, 3.0 g trisodium citrate, 2.0 g KCl, 0.05 g $FeSO_4{\cdot}7H_2O$, and 0.2 mg $MnSO_4{\cdot}xH_2O$, pH 7.4. *Haloarcula*, *Halorubrum*, and *Haloferax* strains were grown in AS-168 medium (per liter): 5.0 g Bacto casamino acids (Difco), 5.0 g Bacto yeast extract (Difco), 1.0 g sodium glutamate, 3.0 g trisodium citrate, 200 g NaCl, 20 g $MgSO_4{\cdot}7H_2O$, 2.0 g KCl, 0.36 g $FeCl_2{\cdot}4H_2O$, and 0.36 mg $MnCl_2{\cdot}4H_2O$, pH ~7.0–7.2. The solution containing the same salts but no casamino acids and yeast extracts was defined as the "basal salt solution", and was used for washing the halobacterial cells when necessary.

Natrinema strains were grown in CM medium (per liter): 7.5 g Bacto casamino acids (Difco), 10.0 g Bacto yeast extract (Difco), 3.0 g trisodium citrate, 200 g NaCl, 20 g $MgSO_4{\cdot}7H_2O$, 2.0 g KCl, and 10 ppm Fe^{2+}, pH 7.5. *Natronobacterium*, *Natrialba*, *Natronomonas*, *Natrononococcus*, and *Natronorubrum* strains were grown in AS-55 medium (per liter): 7.5 g Bacto casamino acids (Difco), 10.0 g Bacto yeast extract (Difco), 3.0 g trisodium citrate, 200 g NaCl, 8.0 g Na_2CO_3, 0.1 g $MgSO_4{\cdot}7H_2O$, 2.0 g KCl, and traces of Fe^{2+} and Mn^{2+}, pH 9.5. The bacterial strains were grown at 37°C in AS-2 medium containing the following components per liter: 10.0 g peptone, 3.0 g beef extract, and 5.0 g NaCl, pH 7.0.

Cultures of halobacteria were grown in liquid media at 37°C with constant shaking (200 rpm), or on 1.2% (w/v) agar plates prepared with the same media in plastic containers. For halocin purification, four 0.8-liter cultures of strain AS7092 were grown in 2-liter flasks, shaking at 120 rpm until the cells reached stationary phase. *Hrr*. *saccharovorum* was used as the indicator strain in all the experiments when halocin activity was detected.

Halocin spectrum and activity assay

To test the activity and spectrum of halocin C8, the indicator strains were grown in appropriate liquid media until stationary phase, and then 0.4 ml of culture was transferred to 20 ml of the same media containing 1.2% (w/v) agar, which had been melted and kept at 50°C, then the plates were poured. In these indicator plates, several wells (1 cm in diameter) were aseptically punched in the lawn of the indicator strain. Next, 0.2 ml culture supernatants from strain AS7092, which contained halocin C8 activity, were loaded in the wells, and the uninoculated AS-169 medium was also loaded in separate wells as control treatments. The plates were then incubated at 37°C in sealed plastic bags. When a clear zone of inhibition appeared around the well, the result was considered as being positive.

To evaluate the levels of halocin activity, two methods were used in this work. For samples with low levels of halocin activity, the activity was given in centimeters according to the following expression: A (cm) = (inhibition halo diameter − well diameter)/2 (Torreblanca et al. 1989). For samples with high levels of halocin activity, a twofold dilution method (Meseguer et al. 1986) was used and the highest dilution producing visible inhibition was considered to contain 1 AU halocin.

Stability assay of halocin C8

Halocin C8 was subjected to the following tests: (1) Heat treatment at about 100°C for 10, 20, 30, 45, or 60 min. (2) Desalting in a MACROSEP centrifugal concentrator with a 3-kDa nominal molecular weight cutoff (NMWCO) spin filter (Pall Filter-Beijing , Beijing, China). After each spin, the retentate was resuspended in 6 volumes of dH_2O and centrifuged again until most salts were washed out. (3) Incubation with proteinase K (100 μg/ml, 400 μg/ml, 1 mg/ml, or 2 mg/ml) and trypsin (100 μg/ml, 400 μg/ml, 1 mg/ml, or 2 mg/ml) for 60 min at 37°C. In this assay, halocin C8 had

been desalted and partly purified. (4) Treatment with equal volume of organic solvents such as methanol, ethanol, isopropanol, and acetonitrile at room temperature for 60 min. The halocin activities before and after each treatment were assayed.

Tangential flow filtration (TFF)

Cultures (3.2 liters) of strain AS7092 were collected at the beginning of the stationary phase. Cells were removed by centrifugation (4,000 rpm, 20 min, twice). Then the supernatants were concentrated using the Vivaflow 50 tangential flow filtration (TFF) system (Vivacience AG, Göttingen, Germany). It was first processed through a 50-kDa NMWCO filter to remove high molecular substances, while approximately 60% of the total activity was passed through. Then the filtrates were concentrated with a 5-kDa NMWCO filter and most activity was retained in a volume of about 15 ml. This material was then divided into two preparations for the following gel filtration chromatography.

Gel filtration column chromatography

The two parts of the retentate from the 5-kDa NMWCO filter were loaded separately onto a Sephadex G50 (1.5–30 kDa NMW range, Pharmacia) column with bed dimensions of 1×110 cm, and eluted with 0.05 M Tris-HCl (pH 8.0) running buffer. The column was run at 8 ml/h, and 2-ml fractions were collected and tested for inhibitory activity. The fractions containing inhibitory activity were combined and then concentrated to 3 ml by freeze-drying. Afterwards, the concentrated samples were reloaded onto a new Sephadex G50 column for an additional gel filtration. After these processes, the halocin C8 was thoroughly desalted and partly purified.

DEAE-sepharose fast flow chromatography

Gel filtration fractions that contained high levels of inhibitory activity were collected, and loaded onto a DEAE-sepharose fast flow column (Pharmacia; with bed dimensions of 1×20 cm) equilibrated with 0.05 M Tris-HCl (pH 8.0). The column was first washed with one-column volume of 0.05 M Tris-HCl (pH 8.0) at 40 ml/h, followed by elution with a two-column volume of the same buffer but containing gradient NaCl from 0.1 to 1.0 M. Several 2-ml fractions of this DEAE-sepharose fast flow chromatography were collected and tested for halocin activity. The fractions containing high levels of halocin activity were combined and concentrated to 2 ml by freeze-drying. Since samples after DEAE-sepharose fast flow column would contain some salts, the concentrated samples were loaded onto the Sephadex G50 column again for desalting, and fractions containing halocin activity were subjected to tricine SDS-PAGE.

Tricine SDS-PAGE and IEF-PAGE

The halocin protein in the column fractions was analyzed by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine SDS-PAGE). The gels consisted of a 16.5% T, 6% C and 13% glycerol separating gel, a 10% T and 3% C spacer gel, and a 4% T and 3% C stacking gel (T, total concentration of both acrylamide and bisacrylamide; C, relative percentage of the bisacrylamide to both monomers). The samples were mixed with equal volumes of 2×loading buffer (Schägger and von Jagow 1987) and incubated at 40°C for 30 min before loading the gel. The gel was run at 30 V for about 1 h until the samples completely entered the stacking gel, and then run for another 4–5 h at 120 V. Proteins in the gel were finally stained with Coomassie Blue.

Isoelectric focusing (IEF) of purified halocin C8 was carried out in polyacrylamide gels with pH gradient using a Bio-Rad Model III mini IEF cell according to the manufacture's instructions (Bio-Rad China, Beijing, China). The standard samples were purchased from Sigma (St. Louis, MO, USA) in a pH range from 3.6 to 9.3. The gel was stained in Coomassie Blue.

N-terminal amino acid sequencing

The purified protein of halocin C8 separated by tricine SDS-PAGE was transferred to BioTrace PVDF membrane (Pall, Ann Arbor, USA), using a Mini-Protean II electro-transfer chamber (Bio-Rad Hercules, CA, USA). The PVDF membrane was stained in Coomassie Blue, and the single band of halocin C8 was cut off and subjected to N-terminal amino acid sequencing by Edman degradation.

Effect of halocin C8 on cells of *Halorubrum saccharovorum*

Samples of 1.5 ml of fresh cultures of *Hrr. saccharovorum* at the early stationary phase were collected by spinning in an Eppendorff tube, and washed twice with 1 ml of basal salt solution. The cells were then incubated with or without halocin C8 (1,280 AU/ml) in the basal salt solution, and treated for 5, 12, 24, and 48 h at 37°C. Afterwards the cells were negatively stained with 1% (w/v) uranyl acetate for 1 min, and the morphological changes were observed with a transmission electron microscope.

Results

Activity spectrum of halocin C8

Halocin C8 appeared to have a wide activity spectrum. It inhibited the growth of 16 out of the 21 tested halophilic archaeon strains, including three alkaliphilic halobacteria that grow optimally at pH 9.5: *Nbt. gregoryi* NCMB2189, *Nab. magadii* NCMB2190, and *Nmn. pharaonis* DSM2160. Only five tested haloarchaeal strains (*Hbt. salinarum* DSM670, *Hfx. denitrificans* ATCC35960, *Nnm. versiforme* JCM10478, *Ncc. occultus* JCM8859, and *Nrr. tibetense* GA33) were insensitive to this halocin. These features much resembled with that of a recently reported microhalocin A4 (O'Connor and Shand 2002). Both C8 and A4 inhibit the growth of the two haloalkaliphilic rods, *Natronobacterium gregoryi*, and *Natronobacterium magadii*, but not haloalkaliphilic cocci such as *Natronococcus occultus*. The difference is that C8 was also active against *Nmn. pharaonis* but A4 was not. However, like all other halocins reported so far, halocin C8 was not able to inhibit any of the bacterial strains tested.

Correlation of halocin C8 activity with the growth phase of strain AS7092

Most halocins were expressed initially at transition to stationary phase (Cheung et al. 1997; Price and Shand 2000; Rdest and Strum 1987; O'Connor and Shand 2002). To test whether it is the case for halocin C8, 2 ml of a fresh culture of strain AS7092 was inoculated into 100 ml AS-169 medium, and incubated at 37°C in an orbital shaker at 200 rpm. Then 0.6 ml of culture was taken out every 6 h for halocin activity assay and cell

concentration determination. Figure 1 represents a typical result of three independent experiments, correlating the growth phase of strain AS7092 with the halocin C8 activity. The results indicated that the halocin activity of C8 was first detected at the transition from exponential to stationary phase, like most other halocins. It increased rapidly to the maximum level within a few hours, and the maximum level of halocin activity remained constant throughout the stationary phase (Fig. 1). This resembles S8 (Price and Shand 2000) but not H4; the activity of the latter rapidly declined to a lower level soon after the maximum level was reached (Cheung et al. 1997).

Stability of halocin C8

There are at least two reasons that halocin can retain a high activity level throughout the stationary phase. One is that the halocin gene may keep expressing, the other is that this halocin may be quite stable. To test these possibilities and develop an appropriate purification scheme, the stability of halocin C8 was comprehensively assayed.

First, the supernatants containing halocin C8 were boiled and sampled periodically for halocin activity assay in order to assess the heat stability. The results showed that halocin C8 did not lose any activity after boiling (at about 100°C) for 1 h. The culture supernatants had also been stored at 4°C, without losing activity, for more than 1 year. These results indicate that halocin C8 is heat-stable. Second, the supernatants containing halocin C8 were desalted with dH_2O using tangential-flow spin concentrator with a 3-kDa NMWCO filter. The desalted halocin C8 had the same activity as the culture supernatant, and could be stored for more than 1 year at −20°C without losing activity. Third, the halocin C8 was treated with organic solvents, and showed that its activity was not affected by methanol, ethanol, and acetonitrile treatments. However, the 50% isopropanol in dH_2O could also produce an inhibitory zone, so it remains to be determined whether isopropanol affected the halocin C8 activity or not. Fourth, the desalted halocin C8 was treated with different proteinases. Results indicated that C8 was sensitive to proteinase K but not to trypsin. Proteinase K (1 mg/ml) could reduce the halocin activity to 50% of the original (10,000 AU/ml) in 1 h. However, its activity was the same before and after trypsin (2 mg/ml) treatment for more than 1 h. These results demonstrate that halocin C8 is a relatively stable peptide.

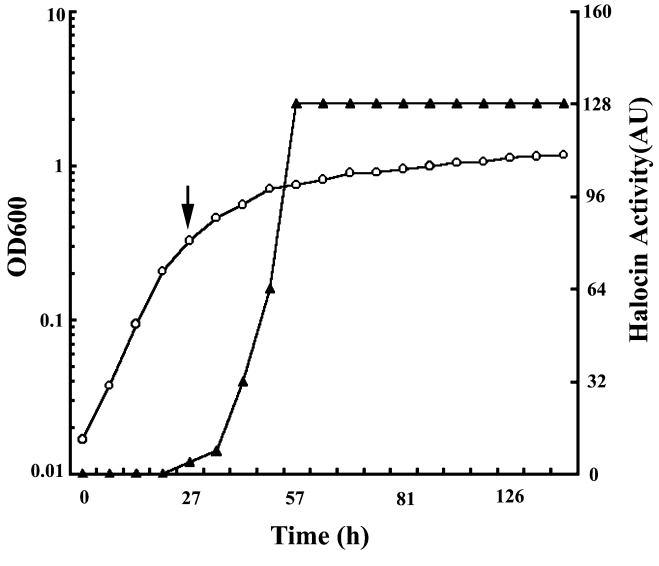

Fig. 1 Correlation of halocin C8 activity with the growth phase of strain AS7092. Growth curve (*circles*) and halocin C8 activity (*triangles*) are shown. *Arrow* shows onset of halocin C8 activity

Purification and molecular-weight determination of halocin C8

Since halocin C8 is quite robust, as it can be desalted, boiled, frozen, subjected to organic solvents, and stored at 4°C for a long period without losing activity, an effective scheme for purification of this halocin was developed, which combined desalting, freeze-drying, gel filtration, and ion exchange chromatography. The supernatants of AS7092 cultures at stationary phase were first concentrated through a series of tangential flow filters with progressively smaller nominal molecular weight cutoffs (NMWC). The fractions retained by a 50-kDa NMWC filter, and that passed through a 50-kDa NMWC filter but retained by a 5-kDa NMWC filter, each contained about 40% of the total halocin activity. SDS-PAGE showed that the 50-kDa retentate contained numerous proteins, including many large and small ones, however, the 5–50 kDa fraction contained rare large proteins. Therefore the 5–50 kDa fraction was then applied to a Sephadex G50 gel filtration column for halocin purification as described in the Materials and methods section. A single activity peak was observed after each gel filtration column chromatography treatment. Since complete desalting had no effect on the inhibitory activity of halocin C8, ion exchange was used afterwards with DEAE-sepharose fast flow, a single activity peak appeared again, and the highest activity was found in one fraction (Fig. 2a). Finally, 2 ml of the concentrated solution with high levels of activity was applied to a Sephadex G50 gel filtration column again. The chromatogram showed two elution peaks but only a single activity peak (Fig. 2b). SDS-PAGE showed that the maximal-activity fraction obtained by this gel filtration column chromatography was electrophoretically homogeneous (Fig. 3a), and the molecular weight of halocin C8 estimated from this SDS-PAGE was about 6.3 kDa.

Determination of the isoelectric point and N-terminal sequence of halocin C8

The purified halocin C8 was first subjected to isoelectric point (pI) determination with IEF-PAGE (Fig. 3b). Its

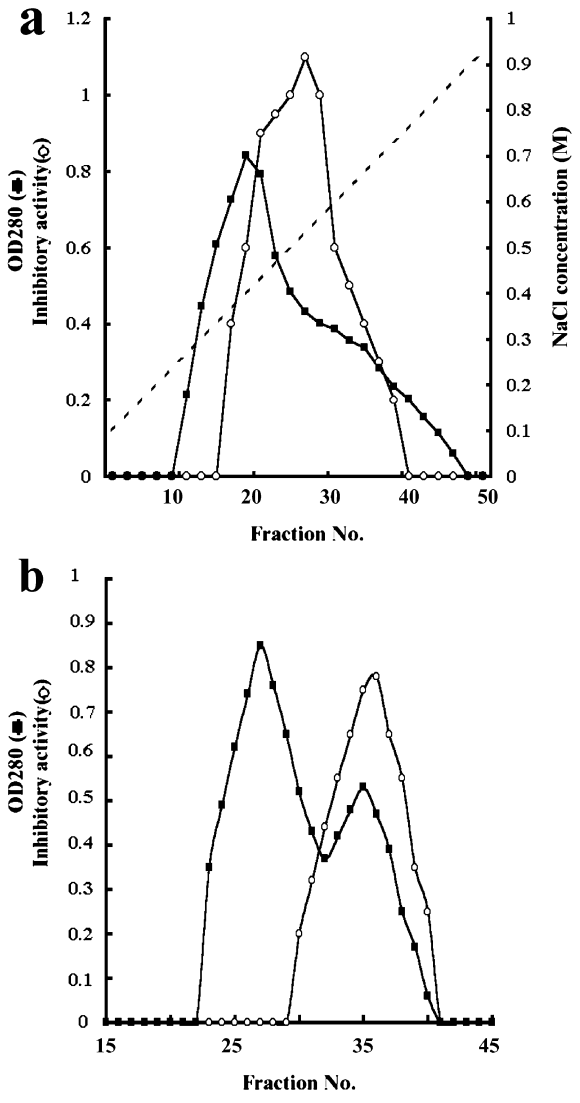

Fig. 2a, b Purification of halocin C8 through column chromatography. **a** Elution profile of halocin C8 from a DEAE-sepharose fast flow column. The OD_{280} (*squares*) and halocin activity (*circles*) of fractions are shown. The NaCl gradient is represented as a *broken line*. **b** Elution profile of halocin C8 from a Sephadex G50 column loaded with concentrated halocin samples after DEAE-sepharose fast flow chromatography. The OD_{280} (*squares*) and halocin activity (*circles*) of fractions are shown. Halocin activity is calculated as *A* (cm) = (inhibitory halo diameter-well diameter)/2 (see Materials and methods)

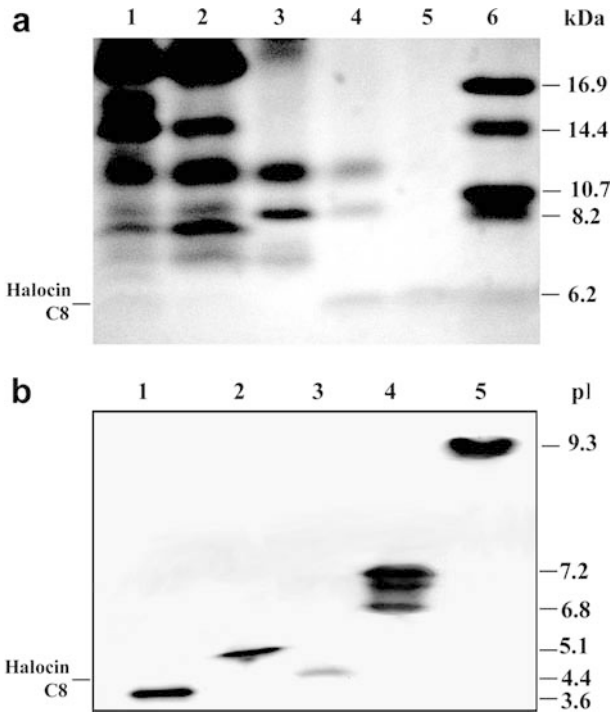

Fig. 3a, b Polyacrylamide gel electrophoresis of purified halocin C8. **a** Tricine SDS-PAGE analysis of samples from each of the purification steps. *Lane 1*, the concentrated proteins in the culture supernatant of AS7092; *lane 2*, concentrated 5-kDa retentate from TFF of culture supernatant; *lane 3*, concentrated samples containing halocin C8 activity from Sephadex G50 column purification; *lane 4*, DEAE-sepharose fast flow fraction containing halocin C8 activity; *lane 5*, purified halocin C8 after the last Sephadex G50 chromatography; *lane 6*, peptide size standards (Pharmacia). Note that the purified halocin C8 is about 6.3 kDa. **b** IEF-PAGE analysis of purified halocin C8. *Lanes 1, 2, 4 and 5* are pI standards (Sigma) and *lane 3* is the purified halocin C8. Note that the pI standards are amyloglusidase (pI = 3.6, lane 1), *β*-lactoglubulin (pI = 5.1, lane 2); myoglobins (two bands, pI = 6.8 and pI = 7.2, lane 4), and trypsinogen (pI = 9.3, lane 5), respectively, and the calculated pI of halocin C8 is about 4.4

homogeneity was further confirmed, as it showed as a single band in the gel. According to the standard proteins with known pI in the same gel, the pI of halocin C8 was calculated as 4.4 (Fig. 3b), so halocin C8 is highly acidic, like most other halobacterial proteins, which usually have high surface negative charges as a major adaptive mechanism in order to function in nearly saturating salinity (Kennedy et al. 2001). This may be also the case for some other halocins. Although the experimental data for pI are currently not available, the theoretical pI values for S8 and H4 can be calculated according to their amino acid sequences (Price and Shand 2000; Cheung et al. 1997) with the "compute pI/Mw tool" (http://us.expasy.org/tools/pi_tool.html). The pI values of S8 and H4 estimated by this software were indeed highly acidic too, at 3.56 and 4.39, respectively.

The N-terminal sequence of purified halocin C8 was also determined with Edman degradation. The first 15 amino acids revealed in the N-terminus of this halocin were D-I-D-I-T-G-C-S-A-C-K-Y-A-A-G. When compared with the N-terminal sequences of S8 and H4, the two halocins sequenced previously, it was shown as a completely novel one with little identity. However, some interesting features were found. All of the three halocins have an acidic amino acid (aspartic acid, D) in the N-terminus in their mature forms. For C8 and H4, the first two amino acids (D and I) are even identical. According to its molecular weight (6.3 kDa), there may be more than 50 amino acids in halocin C8. Since it was hard to completely determine the protein sequence by direct sequencing, the information of the N-terminal

sequence was used to design degenerate DNA probes to clone the corresponding gene (*halC8*), and Southern blotting results showed that this gene seemed to be located in a megaplasmid in the host strain AS7092 (data not shown).

Morphological changes in *Hrr. saccharovorum* treated by halocin C8

To investigate the mechanism of halocin C8 against the sensitive cells, sequential morphological changes of the test strain *Hrr. saccharovorum* that had been exposed to halocin C8 (1,280 AU/ml) were monitored by transmission electron microscope. For the untreated *Hrr. saccharovorum* cells, the morphology was mainly rod-shaped during the entire experimental procedure (Fig. 4a). For the treated cells (Fig. 4b–f), although there were no significant changes observed in the first 2 h, some cells started to swell and showed elliptic or spherical morphology later. The proportion of cells with this shape gradually increased with the extension of exposure time. Afterwards, the cell wall in both poles of some cells was nicked, and the cellular contents were extruded out of the cells (Fig. 4b). The nicks then became larger and more cellular contents flowed away (Fig. 4c). After 24 h, most cells were lysed, with only cell wall debris remaining (Fig. 4d, e). After 48 h, all the cells were completely lysed, with only a few small clumps of electron-dense material in the field (Fig. 4f). Unlike *Hbt. halobium* cells treated by H4 and H6, which induce cell deformation and lysis, leaving empty "ghosts" in which the cell envelopes seem to be intact (Meseguer and Rodriguez-Valera 1986; Shand et al. 1999), the cytoplasm contents of C8-treated *Hrr. saccharovorum* were extruded out, apparently due to cell wall disruption. It is conceivable that the primary target of C8 might be located in the cell wall rather than the cell membrane. However, more data are needed to support this hypothesis.

Discussion

According to the different molecular size, activity spectrum, and N-terminal sequence, halocin C8 seemed to be a novel microhalocin. In addition, it is very robust as it has been shown to be totally insensitive to desalting, boiling, freeze-drying, etc. These properties are advantageous for the development of a very effective purification scheme, e.g., freeze-drying and ion exchange were used to concentrate the samples and to separate the different molecules. Although its ecological role was not assayed, the high stability would help the producer to efficiently inhibit other haloarchaeal species that have the same environmental requirements when resources become exhausted.

Halocin C8 (~6.3 kDa) is the fourth microhalocin so far characterized. The others are halocin S8, R1, and the partially characterized A4 (O'Connor and Shand 2002). Halocin S8 and R1 are cytostatic against sensitive cells, while C8 and A4 are cytocidal. Both C8 and A4 have very broad activity spectra, since in addition to inhibiting most haloarchaea, they are also active against haloalkaliphilic archaeal rods, *Nbt. gregoryi* and *Nab. magadii*, but not the haloalkaliphilic coccus, *Ncc. occultus*. However, halocin C8 also inhibits *Nmn. pharaonis*, but A4 does not. It should be interesting to investigate the protein structural differences that might be responsible for the small differences observed in the activity spectra. Moreover, the morphological changes in the sensitive strain suggest that the primary target of C8 might be located at the cell wall, which is different from H6, whose target is the Na^+/H^+ antiporter in the cell membrane. This observation seems to be in accordance with its activity spectrum. Thus, our results showed that C8 is active against *Natronobacteria* but not *Natronococci*, probably due to the thick natronococcal polysaccharide cell wall with different components. With these new features, halocin C8 should be a good archaeal model for elucidation of the protein structure/function relationship in the future.

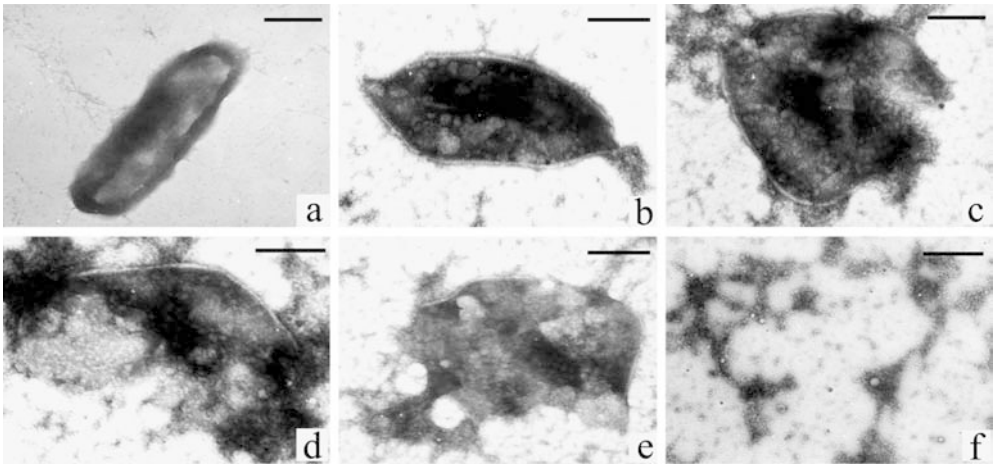

Fig. 4a–f Electron micrographs of *Hrr. saccharovorum* treated with halocin C8. **a** Untreated cells; **b** cells after 5 h exposure to halocin C8; **c** cells after 12 h exposure; **d**, **e** cells after 24 h exposure; **f** cells after 48 h exposure. *Bars* 1 μm

Among the halocins investigated so far, most are first detectable when cultures of the producing cells enter the stationary growth phase (Shand et al. 1999; O'Connor and Shand 2002). In *Hfx. mediterranei* R4, although the transcripts of halocin H4 gene are present at low basal levels during exponential growth, the halocin activity first appears at the end of the exponential growth phase, due to the rapidly increased gene expression level (Cheung et al. 1997). The gene expression style of S8 is similar in H4; however, activity of halocin H4 decreases during the stationary phase, whereas S8 maintains a high activity for a prolonged time (Cheung et al. 1997; Price and Shand 2000). Northern blotting results showed that the transcripts of halocin S8 and H4 genes both decrease to lower levels during the stationary phase, so S8 maintains a high-level activity possibly through its robust stability. In our experiments, the activity of halocin C8 is also first detectable in the cultures at the transition from exponential phase to stationary phase, and the high activity level can also be maintained for a long time during the stationary phase. Although the strong stability should contribute to this feature, the gene expression style of halocin C8 in the stationary phase remains to be investigated. In any case, it adds a new member to the list of the models available to elucidate the regulation of stationary phase gene expression in the haloarchaea.

The aim of this work was to identify novel halocins to explore halocin diversity at both protein and gene expression levels, and to exploit halocins as models for studying protein structure /function, and phase-specific gene regulation. Halocin C8 is a good candidate for this purpose. The gene cloning and protein structure determination of this halocin are under way in our laboratory.

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