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1 **Original article**

2 **The extremely halophilic archaeon *Halobacterium salinarum* ETD5 from the solar**  
3 **saltern of Sfax (Tunisia) produces multiple halocins**

4

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23

24 **Abbreviations**

25 ACN: acetonitrile.

26 ESI: electrospray ionization

27 FPLC: fast protein liquid chromatography

28 HalC8: halocin C8

29 HalHA3: halocin HA3

30 HalS8: halocin S8

31 HalS14: halocin S14

32 HPLC: high performance liquid chromatography

33 LC-MS: liquid chromatography coupled to mass spectrometry

34 MB: Marine broth

35 NBT: 4-nitro-blue tetrazolium chloride

36 PAGE: polyacrylamide gel electrophoresis

37 ROS: reactive oxygen species

38 SDS: sodium dodecyl sulfate

39 SOD: superoxide dismutase

40 SW: sea water

41 TEMED: N, N, N', N'-tetramethylethylenediamine

42 TSB: Trypticase Soy Broth

43 **Abstract**

44 The extremely halophilic archaeon *Halobacterium salinarum* strain ETD5 was previously  
45 isolated from the solar saltern of Sfax (Tunisia) and shown to encode and express halocin S8.  
46 The *Hbt. salinarum* ETD5 culture supernatant was shown here to exhibit high antimicrobial  
47 activity against several halophilic archaea and bacteria of different genera, showing a cross-  
48 domain inhibition. The antimicrobial activity was destroyed by proteases, thus pointing to  
49 halocins. A bioguided purification procedure was applied using two chromatography steps  
50 and antimicrobial assays directed against *Halorubrum chaoviator* ETR14. In-gel screening  
51 assay showed the presence of two antimicrobial bands of approximately 8 and 14 kDa, for  
52 which characterization was investigated by N-terminal sequencing and mass spectrometry.  
53 The full-length form of halocin S8 that contains 81 amino acids and differs from the 36 amino  
54 acid short-length halocin S8 previously described from an uncharacterized haloarchaeon S8a,  
55 was identified in the 8 kDa halocin band. A novel halocin that we termed halocin S14 was  
56 found in the 14 kDa band. It exhibits amino acid sequence identities with the N-terminally  
57 truncated region of the archaeal Mn-superoxide dismutase. These results show that *Hbt.*  
58 *salinarum* ETD5 produces multiple halocins, a feature that had not been described until now  
59 in the domain *Archaea*.

60

61 **Keywords:** Halocins; halophilic archaea; hypersaline environments; *Halobacterium*  
62 *salinarum*; antimicrobial peptides; Mn-superoxide dismutase.

## 63 1. Introduction

64 Microorganisms, which are qualified as extremophiles, are able to colonize extreme  
65 environments that are hostile to life and grow thanks to specific mechanisms of adaptation  
66 they have developed to survive and face up to severe extreme conditions [1]. Hypersaline  
67 environments are extreme habitats that harbor high microbial diversity which varies according  
68 to several environmental factors, including salt concentration [2, 3]. These environments are  
69 either natural ones, such as coastal lagoons, salt deserts, salt lakes, deep submarine basins and  
70 sebkhas, or artificial ones, such as solar salterns built by man for the commercial exploitation  
71 of salt [4]. Previous molecular and metagenomic studies conducted for a thorough  
72 understanding of the biodiversity and ecology of microorganisms within these environments  
73 have shown that microbial life is dominated by members of halophilic archaea belonging to  
74 the class *Halobacteria* [4, 5]. *Halobacteria* require very high salt concentrations, ranging  
75 between 1.5 and 5.8 M NaCl for growth [5].

76 Interestingly, many halophilic archaea have been demonstrated to produce antimicrobial  
77 compounds, among which antimicrobial peptides called halocins [6, 7, 8, 9, 10].  
78 Antimicrobial peptides are universally produced by the three domains of life and act as  
79 defense molecules [11]. They are a key element of the innate immune system in eukaryotes  
80 and particularly mammals [12, 13]. Antimicrobial peptides are involved in microbial  
81 competitions in natural ecosystems. Their production is a common strategy in many Gram-  
82 positive and -negative bacterial genera to grow at the expense of their competitors [14, 15]. In  
83 archaea, several antimicrobial peptides have been identified until now, but only a handful of  
84 them have been characterized [7]. Those include halocins and sulfolobicins produced by  
85 members of the class *Halobacteria* and the order *Sulfolobales*, respectively.

86 The role of halocins in ecosystems is poorly documented and somewhat controversial.  
87 Their production is considered a defense mechanism used by halophilic archaea to inhibit and

88 dominate competitors of the microbial community in hypersaline environments, essentially in  
89 case of nutritional deficiency and lack of niche space [16]. Therefore, these halocins are  
90 active essentially against phylogenetically related microorganisms, as also pointed for  
91 bacteriocins [15], and also against other prokaryotic species living in the same environment.  
92 However, among ninety halophilic archaea and bacteria sampled from different  
93 geographically distant hypersaline environments, antimicrobial interactions were found  
94 mainly between strains from distant locations, suggesting that hypersaline environments in the  
95 world would shelter similar microorganisms with the potential to produce antimicrobial  
96 agents [6].

97 The halocin genes are expressed at the transition between the exponential and stationary  
98 phases of growth [7], except in the case of halocin H1, which is produced during the  
99 exponential phase of growth [17]. Currently, only nine halocins have been either potentially  
100 identified, or produced and partially characterized from different halophilic archaea [7, 10],  
101 while studies have suggested the production of halocins is a universal feature of all members  
102 of halophilic archaea [11, 18]. Among them, only four have been purified and more deeply  
103 characterized. This is presumably due to difficulties in purifying antimicrobial peptides from  
104 halophilic archaea, owing to the presence of the high salt concentrations required in the  
105 cultures, which make their isolation a real challenge compared to other bacteriocins. Those  
106 four halocins are halocin H4 with a molecular mass of 34.9 kDa produced by *Haloferax*  
107 *mediterranei* R4 isolated from a solar saltern in Spain [19], halocin S8 (HalS8) initially  
108 purified from an unidentified haloarchaeal strain S8a, isolated from the Great Salt Lake (Utah,  
109 United States) and described to have a molecular mass of 3.6 kDa [20], halocin C8 (HalC8) of  
110 7.4 kDa purified from *Natrinema* sp. AS7092 isolated from a salt lake in China [21] and  
111 halocin HA3 (HalHA3) of 13 kDa recently shown to be produced by *Haloferax larsenii* HA3  
112 isolated from the Pachpadra salt lake (Rajasthan, India) [10].

113 Previously we have isolated and characterized archaeal and bacterial strains exhibiting  
114 antimicrobial activity against halophilic microorganisms from two ponds of different salinity  
115 in the solar saltern of Sfax. Among them, we have identified two halophilic archaeal strains  
116 *Halobacterium salinarum* ETD5 and *Hbt. salinarum* ETD8, which exhibited a strong  
117 antimicrobial activity of peptidic nature and displayed and expressed the gene encoding  
118 HalS8 [8]. The aim of the present study was to characterize further the peptides associated  
119 with the antimicrobial activity in *Hbt. salinarum* ETD5 using gene analysis, heterologous  
120 expression, as well as peptide purification from culture supernatants and characterization. We  
121 demonstrate the presence of several halocins produced by *Hbt. salinarum* ETD5, among  
122 which the previously identified HalS8 as an 8.6 kDa peptide, and a novel halocin termed  
123 halocin S14 (HalS14) that showed amino acid sequence identities with the archaeal Mn-  
124 superoxide dismutase (Mn-SOD).

125

## 126 **2. Materials and methods**

### 127 *2.1. Archaeal and bacterial strains and growth conditions*

128 Halophilic archaea, *Hbt. salinarum* strain ETD5 (producer strain of HalS8 and HalS14),  
129 *Hbt. salinarum* strain ETD8 (producer strain of HalS8), *Halorubrum chaoviator* ETR14  
130 (target strain for the antimicrobial assays) and *Hrr. chaoviator* ETD3 (control strain for SOD  
131 activity assay) were isolated from a crystallizer pond of the solar saltern of Sfax in Tunisia  
132 (pond TS18; 390 g L<sup>-1</sup> total salts) and cultivated in Sea Water medium 25 (SW 25, 3.4 M  
133 NaCl, pH 7.2, 37°C), as described previously [8]. Other strains from different genera of  
134 halophilic archaea and bacteria, non-halophilic Gram-positive and -negative bacteria used as  
135 target strains for antimicrobial assays are from the collection of the laboratory Molecules of  
136 Communication and Adaptation of Microorganisms (UMR 7245 CNRS-MNHN), or from

137 international collections (Japan Collection of Microorganismes, JCM; Deutsche Sammlung  
138 von Mikroorganismen und Zellkulturen, DSMZ; Centre de Ressources Biologique de  
139 l'Institut Pasteur, CIP). Fungi used as target strains are from the Collection of fungal strains of  
140 the National Museum of Natural History, Paris, France (LCP), which belongs to the European  
141 Culture Collection Organization (ECCO). All these strains are listed in Table 1 [8, 16, 22-45].  
142 For antimicrobial assays, DSMZ Halobacteria medium (M372, 3.4 M NaCl, pH 7, 37°C)  
143 (<http://www.dsmz.de>) was used for the haloarchaeal strains, Trypticase Soy Broth (TSB, 1.7  
144 M NaCl, pH 7, 37°C) (Becton Dickinson) and BD Marine Broth (MB, 1.3 M NaCl, pH 7,  
145 37°C) (Becton Dickinson) for the halophilic bacteria *Nesterenkonia halophila* DSM16378 and  
146 *Halomonas sabkhae* DSM19122, respectively. A malt extract-sodium chloride medium (malt  
147 extract 20 g L<sup>-1</sup>, 1.2 M NaCl, pH 7, 37°C) was used for the antifungal assays.

## 148 2.2. Antimicrobial activity assays

149 The antimicrobial activity of the halocins was detected by the agar-well diffusion assay, as  
150 described previously [8]. The antimicrobial activity was determined after two-weeks  
151 incubation, by the formation of clear inhibition zones around the wells that were measured in  
152 mm.

## 153 2.3. Cultivation of *Hbt. salinarum* ETD5 for production of halocins and treatment of the 154 supernatant

155 Optimal production growth phase of antimicrobial activity by *Hbt. salinarum* ETD5 was  
156 determined previously [8]. Briefly, a stationary stage preculture of strain ETD5 was used to  
157 inoculate a 2-liter culture in SW25 medium supplemented with 3.4 M NaCl. The culture was  
158 incubated for 72 h (stationary phase of growth) at 37°C with continuous 200 rpm shaking and  
159 further centrifuged for 45 min at 8000 g. The crude supernatant (CS) was then dialyzed with



160 Spectra-Por<sup>®</sup> 3, MWCO 3.5 kDa membrane against 1.7 M NaCl for 24 h at 4°C to obtain  
161 dialysed culture supernatant (DCS). The DCS was concentrated under reduced pressure  
162 (Rotavapor<sup>®</sup>) for 3 h at 50°C and a pressure of 60 mPa, until a final volume of 500 mL  
163 (concentrated culture supernatant, CCS) and filtered through 0.22 µm filters (Sartorius), to  
164 generate filtered culture supernatant (FCS). The antimicrobial activity was tested against the  
165 susceptible strain *Hrr. chaoviator* ETR14. The FCS was further subjected either to various  
166 treatments or to the purification procedure (see below).

167 2. 4. *Susceptibility of Hbt. salinarum* ETD5 antimicrobial activity to detergents, variations of  
168 *the NaCl concentration and proteolytic enzymes*

169 The *Hbt. salinarum* ETD5 crude supernatant was used for susceptibility assays of the  
170 antimicrobial agents to the different treatments. After each treatment, the antimicrobial  
171 activity was tested against *Hrr. chaoviator* ETR14.

172 For the susceptibility to detergents, the *Hbt. salinarum* ETD5 supernatant was treated with  
173 Tween-20 or Triton X-100 at different concentrations (1 to 10‰) and incubated for 1 h at  
174 37°C. For the susceptibility to NaCl concentration, the supernatant was dialyzed with  
175 Spectra/Por<sup>®</sup> 6 MWCO 1 kDa membrane against Tris-HCl 10 mM, pH 7 buffer to fully  
176 remove NaCl. Different NaCl concentrations from 0.5 to 4 M were then prepared starting  
177 from the desalted supernatant. For the susceptibility to proteases, the supernatant was treated  
178 with chymotrypsin, thermolysin or endoproteinase GluC (Sigma-Aldrich) at 20 ng µL<sup>-1</sup> in  
179 ammonium bicarbonate buffer 50 mM, pH 8, overnight at 37°C. Antimicrobial assays were  
180 performed as described above.

181 2.4. *DNA sequence of the HalS8 precursor gene*

182 Total DNA was extracted from the cell pellet of a 40 mL stationary phase culture from  
183 *Hbt. salinarum* ETD5, as previously described [8]. PCR reactions were carried out on a Veriti  
184 Thermal Cycler (Applied Biosystems) as previously reported [8]. Oligonucleotide primers  
185 were designed according to the analysis of the sequence of the gene encoding the HalS8  
186 precursor (Genbank AF276080) in the unidentified haloarchaeon S8a [20]: halS8-F (5'-  
187 CGCATATCGGATAAAGACAGC-3') and halS8-R (5'-  
188 GAATTCACCAGAAGCTAGTCCATTCAT-3'). Amplified products were submitted to  
189 electrophoresis on 1% (w/v) agarose gels in Tris-acetic acid-EDTA buffer containing 1X  
190 GelRed® (Biotium, VWR) and visualised under UV light. Amplicons were purified using the  
191 Gene CleanH Turbo kit (MPBio) and cloned into the pGEM-T Easy vector (Promega)  
192 following the manufacturer's instructions. Plasmid DNA from positive clones was extracted  
193 using the QuickLyseH kit (Qiagen, France) and sequenced by Eurofins MWG Operon  
194 (Germany). The sequences were compared with the GenBank database using the BlastN  
195 algorithm. Nucleotide sequence of the *halS8* gene has been deposited under accession number  
196 KR611165.

### 197 2.5. Purification of the *Hbt. salinarum* ETD5 halocins

198 A two-steps procedure including (i) hydrophobic interaction chromatography and (ii) ion  
199 exchange chromatography was applied to the *Hbt. salinarum* ETD5 FCS prepared as  
200 described above. Antimicrobial activity was monitored throughout the purification procedure  
201 using *Hrr. chaoviator* ETR14, as described above. (i) The FCS was submitted to a fast protein  
202 liquid chromatography FPLC (AKTA™ Purifier system, GE Healthcare) using a Phenyl-  
203 Sepharose 6 Fast Flow XK 16/20 column (GE Healthcare) previously equilibrated with 150  
204 mL of 25 mM Tris-HCl, 3.5 M NaCl, pH 8 (buffer A). After injection of 500 mL of the  
205 sample, the column was washed with buffer A to a stable OD at 214 nm and the fractions

206 were eluted at a flow rate of 1 mL min<sup>-1</sup> using a gradient of 0 to 80% acetonitrile (ACN) in  
207 buffer A. The proteins were detected at 214 nm and 1 mL fractions were automatically  
208 collected and submitted to antimicrobial assays using the agar well diffusion method. Active  
209 fractions were concentrated to 0.5 mL using a SpeedVac® (Savant) and further pooled. (ii) 1  
210 mL of the active fractions obtained from the Phenyl-Sepharose step was loaded onto a  
211 MonoQ HR 5/5 column (GE Healthcare) previously washed with 5 mL of 25 mM Tris-HCl  
212 buffer, pH 8 (buffer B) and equilibrated with 10 mL of 25 mM Tris-HCl buffer, 1.5 M NaCl,  
213 pH 8. The column was washed with 5 mL of buffer B before injection of the sample and  
214 fractions were eluted at a flow rate of 0.25 mL min<sup>-1</sup> with a step-increasing gradient from 25  
215 to 500 mM NaCl. The proteins were detected at 214 nm and 1 mL fractions were  
216 automatically collected and concentrated using a SpeedVac® (Savant). The fractions were  
217 stored at 4°C until analysis.

## 218 2.6. *Polyacrylamide gel electrophoresis and antimicrobial in-gel assay*

219 The purification fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel  
220 electrophoresis (SDS-PAGE) on 17% SDS Tris-tricine polyacrylamide gels. PageRuler low  
221 range unstained protein ladder (Fermentas) or Ultra-low range molecular weight marker  
222 (Sigma-Aldrich), or low range rainbow molecular weight marker (GE Healthcare) were used  
223 as molecular weight standards. For the in-gel assay, 20 µL of a purified fraction were loaded  
224 onto two lanes of a 17% SDS Tris-tricine polyacrylamide gel. Following separation, the first  
225 part of the gel containing the PageRuler molecular weight marker was fixed with 40% (v/v)  
226 methanol, 7% (v/v) acetic acid and stained with silver nitrate. After staining, the gel was  
227 visualized using a Fusion-FX7 system (Viber Lourmat). The second part of the gel containing  
228 the colored low range rainbow molecular weight marker was fixed for 30 min in 15% (v/v)  
229 isopropanol and 10% (v/v) acetic acid and washed with MilliQ® water (Merck Millipore)

230 overnight. This unstained gel was then deposited on SW agar and overlaid with 10 mL SW  
231 soft agar inoculated with 100  $\mu$ L of a *Hrr. chaoviator* ETR14 preculture at the end of the  
232 exponential phase of growth. Plates were incubated at 37°C for two weeks and examined for  
233 inhibition halos.

#### 234 2.7. SOD assay

235 Intracellular protein extracts were obtained from cultures of *Hbt. salinarum* ETD5, *Hbt.*  
236 *salinarum* ETD8 or *Hrr. chaoviator* ETD3, in 50 mL of SW 25 medium. After centrifugation  
237 of 5 mL of the culture, cell pellets were resuspended in 2 mL of lysis buffer (25 mM Tris-HCl  
238 2 mM EDTA, pH 7.6) supplemented with 1.7 M NaCl. The mixtures were incubated with  
239 0.25 mg.mL<sup>-1</sup> lysozyme (Sigma-Aldrich) for 20 min at room temperature. The cell walls were  
240 destroyed by ultrasound sequences (8 times for 15 s at 30% amplitude in ice) using a  
241 sonicator (Branson Digital Sonicator). Cell debris were centrifuged for 10 min at 10 000 g.  
242 The SOD activity was revealed on a 12% gel under native conditions containing riboflavin  
243 (Sigma-Aldrich) and 4-nitro-blue tetrazolium chloride (NBT) (Sigma-Aldrich), according to a  
244 protocol optimized from [46] and [47]. After 3 h of 90 V pre-electrophoresis in 10%  
245 migration buffer (0.19 M Tris-HCl, 1 mM EDTA, pH 8.8) to remove N, N, N', N'-  
246 tetramethylethylenediamine (TEMED) and ammonium persulfate, either 100 ng of  
247 intracellular proteins extracted from the cell pellets of *Hbt. salinarum* ETD5, *Hbt. salinarum*  
248 ETD8 or *Hrr. chaoviator* ETD3 used as control, or the Phenyl-Sepharose fraction were  
249 submitted to electrophoresis. The gel was first stained with a solution containing 2.45 mM  
250 NBT, 1.7 M NaCl, 50 mM sodium phosphate buffer, pH 7.4 for 15 min in the dark. The  
251 solution was emptied and the gel was further stained with a second solution of 28 mM  
252 TEMED and 0.06 mM riboflavin in the same buffer and incubated for 15 min in the dark.  
253 Finally, the gel was illuminated with a fluorescent lamp for 15 min to initiate the

254 photochemical reaction. All procedures were carried out at room temperature and the SOD  
255 positive bands were visualized as clear white regions in the blue formazan background.

#### 256 2.8. *Extraction of halocins from SDS-PAGE gels*

257 The 8 kDa- and 14 kDa-bands from the Phenyl-Sepharose purified fractions were excised  
258 from an SDS-PAGE gel stained with Blue Safe (LabSafe™ Gel Blue, G-Biosciences),  
259 washed with MilliQ® water (Merck Millipore), ground and Speedvac® (Savant) dried for  
260 Edman sequencing and mass spectrometry analysis. For Edman sequencing, the bands were  
261 incubated at 37°C overnight with agitation in 100 mM Tris-HCl, 3.5 mM SDS, 100 mM  
262 NaCl, pH 8 transfer buffer. A polyvinylidene fluoride membrane was activated by methanol  
263 for 60 s, rinsed with MilliQ® water (Merck Millipore) and incubated with the bands at 37°C  
264 for 24 h for peptide transfer before being submitted to protein sequencing. For mass  
265 spectrometry, the bands were excised from the gel and subjected to reduction and alkylation,  
266 using two successive incubations with 10 mM DTT (Sigma-Aldrich) for 1 h at 57°C and 55  
267 mM iodoacetamide (Sigma-Aldrich) for 1 h at room temperature in the dark. The bands were  
268 then treated as described previously [48], with the exception that the digestion was performed  
269 with chymotrypsin (Sigma-Aldrich) at 20 ng  $\mu\text{L}^{-1}$ . The digests were analyzed by liquid  
270 chromatography - mass spectrometry (LC-MS).

#### 271 2.9. *Edman sequencing*

272 N-terminal sequencing was carried out by automated stepwise Edman degradation using a  
273 Procise Model 492 protein sequencer (Applied Biosystems) according to the manufacturer  
274 protocol.

#### 275 2.10 *Mass spectrometry*

276 Sequence analyses of the chymotrypsin digests of the 8 kDa- and 14 kDa-bands were  
277 conducted by LC-MS/MS on an Ultimate U3000 Micro-LC system (Thermo Scientific)  
278 connected to an ESI-Q-TOF mass spectrometer (Q-STAR Pulsar, Sciex). The separation was  
279 carried out on a C18 Uptisphere 5WTF micro-column (1x150 mm, 5  $\mu$ m, Interchim), using  
280 the following gradient of mobile phase A (ultra-pure water/0.1% formic acid) and B (HPLC-  
281 grade acetonitrile/0.08% formic acid) over a total run time of 55 min: linear increase from  
282 10% B to 60% B for 40 min, linear increase to 100% B for 5 min, decrease to 10% B for 1  
283 min, at a flow rate of 40  $\mu$ L min<sup>-1</sup>. The MS instrument was operated in positive ion mode  
284 using information-dependent acquisition, a mode which completes a series of 1-s survey scans  
285 ( $m/z$  detection range 250–1,300) followed by two 2-s MS-MS experiments on the two most  
286 intense peaks from the survey scan, as long as they have not been fragmented in the last 2 min  
287 ( $m/z$  detection range 50–500). The data generated were analyzed with MASCOT MS-MS ion  
288 search engine [49]. For precise mass measurement of the 8 kDa-band, the samples were  
289 analyzed by LC-MS on a ultra-high performance LC system (UHPLC, Ultimate 3000 RSLC,  
290 Thermo Scientific) connected to a high-resolution ESI-Q-TOF mass spectrometer (Maxis II  
291 ETD, Bruker Daltonics). Separation was achieved on a Jupiter C4 column (1x250 mm, 5  $\mu$ m,  
292 300 Å, Phenomenex) at a flow rate of 200  $\mu$ L.min<sup>-1</sup>, using the following gradient of solvent A  
293 and solvent B' (HPLC-MS-grade acetonitrile/0.08% formic acid) over a total run time of 22  
294 min: isocratic elution with 5% B' for 2 min, linear increase from 5% to 95% B' for 10 min,  
295 isocratic elution with 95% B' for 3 min, linear decrease to 5% B' for 0.1 min. The MS spectra  
296 were acquired in positive ion mode in the mass range  $m/z$  300–3000. The source parameters  
297 were as follows: nebulizer gas 26 psi, dry gas 8 L/min, capillary voltage 4500 V, end plate  
298 offset 500 V, temperature 200°C.

299

### 300 **3. Results**

301 3.1. *Spectrum of activity of Hbt. salinarum ETD5*

302 The extreme halophilic *Hbt. salinarum* ETD5 isolated from the crystallizer pond TS18 of  
303 the solar saltern of Sfax has been shown previously to inhibit the growth of several halophilic  
304 strains from the same environments [8]. We examined further the spectrum of activity of this  
305 strain by antimicrobial assays conducted with the ETD5 culture supernatant against various  
306 halophilic archaea, halophilic and non-halophilic bacteria and fungi (Table 2). *Hbt. salinarum*  
307 ETD5 exhibited a broad spectrum of activity directed against both several halophilic archaea  
308 of different genera, and particularly *Halobacterium*, *Haloarcula*, *Haloterrigena* and to a  
309 lesser extent *Natrinema*, as well as against halophilic bacteria, such as *Salinibacter ruber* and  
310 *Lentibacillus halophilus*. Unexpectedly, one non-halophilic human pathogen, *Pseudomonas*  
311 *aeruginosa* was inhibited by *Hbt. salinarum* ETD5 supernatant. Antimicrobial assays  
312 performed against other non-halophilic bacteria and halophilic fungi were negative. At this  
313 stage, the results point that *Hbt. salinarum* ETD5 has a broad spectrum of activity that not  
314 only targets tightly related archaea.

315 3.2. *Effects of proteolytic enzymes, NaCl concentration and detergents on the Hbt.*  
316 *salinarum ETD5 antimicrobial activity*

317 The sensitivity of the *Hbt. salinarum* ETD5 antimicrobial activity to proteolytic enzymes,  
318 NaCl concentration and detergents was examined to both estimate the stability of the potential  
319 halocins toward these factors and direct the optimization of the purification procedure. The  
320 activity was assayed against *Hrr. chaoviator* ETR14, an haloarchaeon we previously isolated  
321 from the saline of Sfax with optimal sensitivity in the antagonistic assays [8] (Table 3).

322 Treatments of the *Hbt. salinarum* ETD5 supernatant with chymotrypsin, thermolysin or  
323 endoproteinase GluC resulted in a total loss of activity against *Hrr. chaoviator* ETR14,  
324 indicating unambiguously the proteinaceous nature of the compounds involved. It could thus

325 be deduced that the production of one or several halocins was related to the *Hbt. salinarum*  
326 ETD5 activity. As regard the effects of the NaCl concentration, the inhibition halos were  
327 significantly lower (from 32 [ $\pm$  0.8] mm to 18 [ $\pm$  1.6] mm) upon dialysis against MilliQ<sup>®</sup> water  
328 (Merck Millipore) and 0.5 M NaCl was not sufficient to restore the initial activity (Table 3).  
329 Increasing NaCl concentration allowed better antimicrobial activity, with an optimum  
330 between 1.5 and 4 M NaCl (Table 3). Such a requirement of a NaCl concentration threshold  
331 to remain active is a characteristic already described for other halocins [7]. Finally, the ETD5  
332 supernatant was treated with detergents at concentrations between 1‰ and 10‰. Significant  
333 lower inhibition halos were observed with the different concentrations of Tween-20 or Triton  
334 X-100. They were only 12 ( $\pm$  1.6) and 13 ( $\pm$  0.8) mm in diameter at 10‰ Tween 20 and  
335 Triton X-100, respectively, compared to the control untreated supernatant which exhibited a  
336 32 ( $\pm$  0.8) mm diameter (Table 3). Sensitivity of the supernatant activity to proteases suggests  
337 that the antimicrobial compounds produced by *Hbt. salinarum* ETD5 can be attributed to  
338 halocins. In addition, these results point that detergents have to be prohibited for their  
339 purification.

### 340 3.3. Sequence of the *HalS8 precursor gene* *halS8*

341 We had previously shown that *Hbt. salinarum* ETD5 encodes and expresses HalS8 [8], but  
342 the complete *halS8* gene had not been sequenced. To determine the predicted amino acid  
343 sequence of the HalS8 antimicrobial peptide, the complete sequence of the *halS8* gene was  
344 determined here. PCR amplification using primers designed to be localized upstream and  
345 downstream the nucleotide sequence of *halS8* that encodes the HalS8 precursor and the DNA  
346 extracted from *Hbt. salinarum* ETD5 as template, showed amplification of a fragment of 1824  
347 bp. The amplicons were cloned and sequenced. The translation of the 1824 bp-fragment  
348 exhibited an open reading frame of the expected size (936 bp) for the gene encoding the



349 HalS8 precursor. Analysis of the predicted complete amino acid sequences confirmed that the  
350 genome of *Hbt. salinarum* ETD5 contains a complete *halS8* gene, with an amino acid  
351 sequence almost identical to that of strain S8a, initially described as producing HalS8 [20].  
352 Along the 311 residues of the HalS8 precursor, a single substitution of a valine at position 185  
353 in strain S8a for isoleucine in *Hbt. salinarum* ETD5 was observed (Fig. 1). Moreover, the  
354 amino acid sequence deduced from the *halS8* gene located on megaplasmid 2 of  
355 *Halobacterium* sp. GN101 [7] which has been shown to produce halocin R1 [18], is identical  
356 to that deduced from the *halS8* gene sequence of *Hbt. salinarum* ETD5 (Fig. 1).

#### 357 3.4. *Pre-purification of halocins from Hbt. salinarum* ETD5 cultures

358 All attempts for heterologous expression of HalS8, either in a halophilic archaeal strain,  
359 *Haloferax volcanii* H98 [50] or in *Escherichia coli* were unsuccessful (data not shown). In  
360 parallel, classical methods for production and purification of the halocins produced by *Hbt.*  
361 *salinarum* ETD5 were conducted. Optimal culture conditions in terms of culture medium and  
362 phase of growth for maximal antimicrobial production by strain ETD5 determined previously  
363 [8] were applied. Cultures of 0.5 to 2 L grown in SW25 medium supplemented with 3.4 M of  
364 NaCl were used to compare the efficiency of several chromatography techniques in permitting  
365 the isolation of the antimicrobial peptides. Solid phase extraction on SepPak® C18 cartridges  
366 of the FCS showed activity in the 80% ACN fraction only, pointing the highly hydrophobic  
367 nature of the peptides. Reversed-phase C8 HPLC, gel filtration on Sephadex G25, or  
368 chromatography on Phenyl Sepharose were applied, but only the Phenyl Sepharose procedure  
369 allowed recovering a significant activity (data not shown). It was thus retained as the first step  
370 of purification and for obtaining a pre-purified halocin fraction.

#### 371 3.5. *Purification of halocins*

372 *Hbt. salinarum* ETD5 halocins were purified in a two-step procedure including successive  
373 hydrophobic interaction and anion exchange chromatographies, using Phenyl Sepharose and  
374 MonoQ columns, respectively. The purification was guided by antimicrobial assays against  
375 *Hrr. chaoviator* ETR14. At the level of the first step (Supplemental Fig. S1A and  
376 Supplemental Fig. S1C), 27 fractions which revealed antimicrobial activity with diameters of  
377 8 to 22 mm, were further pooled and named fraction P3. SDS-PAGE analysis on a 17%  
378 polyacrylamide gel and silver nitrate staining revealed a quite complex mixture of proteins,  
379 among which a major one around 8 kDa, several others between 12 and 14 kDa and one at  
380 about 24 kDa. Two of the bands, which were observed at approximately 8 and 14 kDa (Fig.  
381 2A), led to a strong inhibition of the growth of *Hrr. chaoviator* ETR14 overlaid in an in-gel  
382 screening assay (Fig. 2B). No band was observed below 8 kDa, thus excluding the presence of  
383 significant amounts of a peptide of 3.6 kDa that could correspond to the 36-residue HalS8  
384 previously described [20].

385 A second purification step was carried out on the fraction P3 by anion exchange  
386 chromatography (Supplemental Fig. S1B). The antimicrobial assays revealed only two active  
387 fractions, E6 and E7. When analyzed on Tris-tricine SDS-PAGE gel stained with silver  
388 nitrate, two bands at about 8 and 14 kDa of almost similar intensity were observed in fraction  
389 E6, while a main band at about 14 kDa was observed in fraction E7 (Fig. 2C). The 8 kDa- and  
390 14 kDa-bands, corresponding to proteins with antimicrobial activity as detected with the in-  
391 gel assay, corresponded to halocins. These active fractions highly enriched in halocins were  
392 used for further characterization.

### 393 3.6. Characterization of the 8 kDa halocin band

394 Edman sequencing identified SDCNINXNT as the nine N-terminal amino acids of the 8  
395 kDa halocin band (Fig. 3A). They were in agreement with those previously determined by

396 Price and Shand for the HalS8 peptide from the unidentified strain S8a [20]. Undigested 8  
397 kDa halocin band excised from SDS-PAGE gel was analyzed by LC-MS (Fig. 4), which  
398 revealed  $[M+nH]^{n+}$  species with charge states 8+ to 13+. This corresponded to the theoretical  
399 mass of 8589 Da calculated for a full-size 81 amino acid HalS8 containing 8 oxidized  
400 cysteines, as expected from the amino sequence deduced from the precursor nucleotide  
401 sequence. Consequently, as opposed to HalS8 from strain S8a, the amino acid sequence of  
402 HalS8 from *Hbt. salinarum* ETD5 did not appear to involve a cleavage of the precursor in the  
403 C-terminal region (Fig. 3A). This result suggests that HalS8 is produced by *Hbt. salinarum*  
404 ETD5 under a full-size 81 amino acid peptide, while the 36 amino acid peptide described by  
405 [20] from strain S8a is not detected in strain ETD5. To analyze further the amino acid  
406 sequence of the 8 kDa halocin band, the chymotrypsin digest was also analyzed by LC-  
407 MS/MS. However, no peptide corresponding to HalS8 could be clearly identified, while  
408 analysis revealed peptides assigned to flagellin B2 and an hypothetical protein VNG\_2044H  
409 deduced from the genome of *Halobacterium salinarum* NRC-1 (data not shown). The lack of  
410 detection of HalS8 chymotryptic peptides could be due to its compact structure linked to the  
411 presence of 4 disulfide bridges, which would not allow chymotrypsin to access its digestion  
412 target sites, despite the reduction and alkylation treatments we used before digestion.

### 413 3.7. Characterization of the 14 kDa halocin band

414 Edman sequencing identified the twelve first amino acids GALGDVQXNGII in the N-  
415 terminal region of 14 kDa halocin band from the SDS-PAGE gel (Fig. 3B). Analysis of the  
416 chymotrypsin digest by LC-MS/MS allowed identifying the sequences of three peptides (Fig.  
417 3B), for which homology searches in the GenBank database by BlastP algorithm revealed  
418 similarity to the 200 residue Mn-superoxide dismutase (Mn-SOD) of *Hbt. salinarum* NRC-1  
419 (UniProt n° P09737) (Fig. 4B). Remarkably, the N-terminal sequence identified by Edman

420 analysis also presented 75% identity to a sequence found in the Mn-SOD. Such a N-terminal  
421 end would yield a protein of approximately 14 kDa, consistent with the mass on SDS-PAGE.  
422 Besides the Mn-SOD peptides, we also found digestion peptides assigned to flagellins and the  
423 hypothetical protein VNG\_2044H deduced from the genome of *Halobacterium salinarum*  
424 NRC-1 (data not shown), as also observed for the 8 kDa halocin band, which were attributed  
425 to contaminants.

426 Taken together these results suggest that the 14 kDa halocin band, to which antimicrobial  
427 activity is associated, contains a novel halocin that presents similarities to Mn-SOD enzymes,  
428 and that we termed HalS14. It appears that one of the four manganese-binding sites of the  
429 catalytic site of Mn-SOD from *Hbt. salinarum* NRC-1, which is located in the N-terminal  
430 region is lost in the HalS14 amino acid sequence (H29), while the three other sites (H73,  
431 D158, H162) are still present (Fig. 3B).

### 432 3.8. Antioxidant and antimicrobial activities of *Hbt. salinarum* ETD5

433 Because HalS14 amino acid sequence displayed similarities to Mn-SOD enzymes from  
434 other halophilic archaea and particularly *Hbt. salinarum* NRC-1, the SOD activity was tested.  
435 The fraction from Phenyl-Sepharose enriched in HalS14, and the whole intracellular proteins  
436 extracted from cell pellets of *Hbt. salinarum* ETD5, *Hbt. salinarum* ETD8 and *Hrr.*  
437 *chaoviator* ETD3 [8, 51] were submitted to the NBT assay (Supplemental Fig. S2). Native gel  
438 electrophoresis showed that together with *Hrr. chaoviator* ETD3 and *Hbt. salinarum* ETD8,  
439 *Hbt. salinarum* ETD5 whole intracellular protein extract exhibited strong SOD activity  
440 (Supplemental Fig. S2). By contrast, the HalS14-enriched fraction did not display such  
441 activity (Supplemental Fig. S2). Parallel determination of the antimicrobial activity showed it  
442 strongly inhibited *Hrr. chaoviator* ETR14 (data not shown). Altogether these results indicate  
443 that whole intracellular proteins from *Hbt. salinarum* ETD5 exhibit both SOD and

444 antimicrobial activities, in agreement with the presence of the SOD enzyme in strain ETD5,  
445 and that HalS14 is associated to the antimicrobial activity against strain ETR14, but devoid of  
446 SOD activity.

447

#### 448 4. Discussion

449 This study aimed at identifying the halocins responsible for the antagonistic properties  
450 exhibited by the extreme haloarchaeon *Hbt. salinarum* ETD5 isolated from the crystallizer  
451 pond TS18 in the solar saltern of Sfax [8]). Here we used a procedure optimized for *Hbt.*  
452 *salinarum* ETD5 that associated dialysis of the supernatant to decrease NaCl concentration  
453 until reaching the threshold that allows the activity to be preserved, concentration under  
454 reduced pressure, and a separation protocol using hydrophobic interaction and ion exchange  
455 chromatographies. However, due to low production yields of the antimicrobial peptides and  
456 the lack of usable archaeal tools for obtaining recombinant halocins in spite of tenacious  
457 efforts, we only obtained tiny amounts of partly purified HalS8 and HalS14 that were used for  
458 further characterization.

459 The complete amino acid sequence of the HalS8 precursor determined in this study shows  
460 identity with predicted amino acid sequences of the HalS8 precursors from the  
461 uncharacterized haloarchaeon S8a isolated from the Great Salt Lake [20] and *Halobacterium*  
462 sp. GN101, with only one substitution among the 311 residues. In a pioneer study, HalS8 was  
463 identified as a 36 amino acid peptide produced by strain S8a [20]. This short-length form of  
464 the halocin would require a double proteolytic processing of the 311-amino acid precursor  
465 encoded by the gene *hals8* [7]. The first processing would occur at an N-terminal site to  
466 release a leader peptide, similar to what is usually observed for bacteriocins and microcins  
467 [52]. The second processing would release the 45 amino acid C-terminal region. In  
468 bacteriocins and archaeocins, a double precursor processing leading to two fragments with

469 different functions is a rare and original feature. It was observed for the first time for HalC8,  
470 which maturation leads both to the halocin and the immunity protein that protects the strain  
471 against its antimicrobial toxin [21]. The mature 76 residue HalC8 is produced by release from  
472 the N-terminus of a 40 amino acid Tat signal peptide, followed by a 167 amino acid immunity  
473 protein, and there is no cleavage at the C-terminus. Therefore, similar to what happens for  
474 HalC8, it could be hypothesized here that the 230 amino acid N-terminal region released from  
475 the HalS8 precursor could contain a short leader or signal peptide, followed by an immunity  
476 protein.

477 Here we have shown that in *Hbt. salinarum* ETD5 HalS8 is likely produced under a full-  
478 length 81 amino acid peptide that results from a single proteolytic cleavage. Since the  
479 maturation processes observed in strains S8a and *Hbt. salinarum* ETD5 appear distinct, the  
480 question is raised of the hypotheses that could explain the occurrence of two different  
481 maturation processes leading to two different forms of this halocin, depending on the  
482 archaeon strain. This could either come from i) a different maturation in the two strains  
483 having different genetic and ecological backgrounds, ii) a different growth state of the strains  
484 when the cultures were harvested that could favor a single or a double processing, or iii) a  
485 purification process that could induce the supplementary C-terminal cleavage or favor the  
486 purification of one form over the other. Further work comparing the two strains would bring  
487 data allowing to strengthen either of these hypotheses and evidence if two different  
488 maturation processes can coexist in halophilic archaea for the production of short-length  
489 or/and longer forms of a given halocin.

490 The newly identified halocin HalS14 exhibits amino acid sequence similarities to the Mn-  
491 SOD of *Hbt. salinarum* and its molecular weight, as determined on SDS-PAGE, is consistent  
492 with a proteolytic processing of the Mn-SOD at the N-terminal extremity. To cope with harsh  
493 conditions and combat reactive oxygen species (ROS) halophilic archaea use enzymatic

494 degradation systems, among which SOD. These metalloenzymes catalyze the dismutation of  
495 the superoxide anion to dioxygen and hydrogen peroxide [53, 54]. The presence and  
496 expression of genes encoding proteins of the SOD family are found in various species of  
497 halophilic archaea [55]. Interestingly, the genomes of *Halobacterium* strains were found to  
498 contain two copies of divergent paralogous SOD-encoding genes, among which the  
499 expression of only one was induced by oxidative stress [56]. In *Hbt. salinarum* ETD5, SOD  
500 would be produced to play this protecting role against ROS, but also it would be processed to  
501 produce HalS14. Alternatively, HalS14 may be derived from the expression of the copy of the  
502 SOD gene that is not dependent on oxidative stress. Thus, HalS14 may contribute to the  
503 competition strategies in concert with HalS8. This could explain the higher antimicrobial  
504 activity exhibited by *Hbt. salinarum* ETD5 compared to *Hbt. salinarum* ETD8, a strain we  
505 identified in the same crystallizer pond of the saline of Sfax and that also expresses the *hals8*  
506 gene similar to *Hbt. salinarum* ETD5 [8]. The production of more than one bacteriocin, with  
507 two or more being quite common, has already been described in both Gram-positive and -  
508 negative bacteria [57, 58, 59]. However, there is little understanding of the reasons why  
509 multiple production of bacteriocins and microcins encoded in the same bacterial strain is not  
510 so rare. It could increase the competitiveness of the strain, confer a selective advantage in  
511 different environments, or decrease the evolution of resistance to the bacteriocin [60], but  
512 none of these hypotheses has been tested experimentally. By contrast, and although it was  
513 suggested previously [9, 61], this is the first time in this study that such a co-occurrence of  
514 several archaeocins in a single halophilic archaeal strain is clearly evidenced. Therefore,  
515 strain ETD5 would be particularly well equipped to occupy the niche and outcompete many  
516 other haloarchaeal strains in the solar saltern. This could explain the broad spectrum of  
517 activity of the strain, which inhibits the growth of different genera of halophilic archaea  
518 (*Halobacterium*, *Haloarcula*, *Natrinema*, *Haloterrigena*) and bacteria (*Salinibacter* and

519 *Lentibacillus*). This result is interesting, because halocins are expected to be active essentially  
520 on closely related archaea, although cross-domain inhibitions have been documented in  
521 previous studies [6]. In this study, *Hbt. salinarum* ETD5 culture supernatant also appeared  
522 capable of inhibiting *P. aeruginosa*. However, to be able to estimate the importance of this  
523 result, it is necessary first to delineate the respective roles of HalS8 and HalS14, and other  
524 potential antimicrobial compounds in the activity against this pathogen.

525 HalS14 may derive from a proteolytic processing of the *Hbt. salinarum* Mn-SOD that  
526 would eliminate an N-terminal 60-residue region to lead to the 140 amino acid antimicrobial  
527 peptide HalS14. Such a feature, an antimicrobial peptide resulting from the processing of a  
528 major protein endowed with a vital function would represent a completely novel finding in  
529 archaea. By contrast, few examples among a lot can be reported in eukaryotes. A short  
530 antifungal peptide was found produced in the shrimp *Litopenaeus vannamei* by cleavage of  
531 hemocyanin [62], the respiratory pigment serving the role of hemoglobin in crustaceans.  
532 Similarly, antimicrobial peptides derive from hemoglobin in mammals [63]. Other  
533 antimicrobial peptides are processed from histones [64], milk proteins [65], or matrix proteins  
534 [66]. Interestingly, the C-terminal region of the human extracellular SOD leads to a cationic  
535 heparin-binding peptide that shows a broad antimicrobial spectrum against bacteria and fungi  
536 [67]. It is the first time here that such a processing, which is to our knowledge not observed in  
537 bacteria, could be hypothesized in archaea, similarly to eukaryotes.

538 In conclusion, this study has demonstrated for the first time the co-occurrence of different  
539 halocins that could act in concert in a haloarchaeal strain. We have shown that HalS8 is  
540 produced by *Hbt. salinarum* under its full-length 81 amino acid peptide. Furthermore, we  
541 showed that the second halocin, HalS14 could derive from the *Hbt. salinarum* SOD through a  
542 specific proteolytic cleavage, which would make a major *Halobacterium* protein endowed  
543 with a vital function in the archaeon also able to ensure a function of defense or competition.



544 Further studies will have to evaluate if the two halocins have different and complementary  
545 spectra of activity and if they act in synergy or not, in order to better appreciate their roles in  
546 nature. Taken together these results show that the *Hbt. salinarum* strain ETD5 is particularly  
547 well equipped for microbial competitions and open new tracks to deeper determine the  
548 importance and the roles of microorganisms in extreme environments and particularly those  
549 of halophilic archaea, which are a source of active biomolecules with many functions, and  
550 particularly antimicrobial and antioxidant factors.

### 551 **Conflict of interests**

552 The authors declare that they have no conflict of interest.

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563

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749 **Figure legends**

750 Fig. 1. Alignments of the amino acid sequences of HalS8 precursors from the uncharacterized  
751 haloarchaeon S8a (Genbank accession N° Q9HHA8, Price and Shand 2000), *Halobacterium*  
752 sp. GN 101 (Genbank accession n° EU080936, [7]) and *Hbt. salinarum* ETD5 (Genbank  
753 accession n° ALF62560, this study). The amino acid substitutions are pointed in shaded dark  
754 grey. Sequences of mature HalS8 following [20] (36 amino acids) and determined in this  
755 study (81 amino acids) are underlined and in bold character, respectively. Stars indicate the  
756 eight cysteines in the HalS8 amino acid sequence from *Hbt. salinarum* EDT5.

757 Fig. 2. Tris-tricine SDS PAGE and detection of the antimicrobial activity of the fractions  
758 eluted from the Phenyl-Sepharose and the MonoQ columns. (A) Analysis of the Phenyl-  
759 Sepharose fractions, gel stained with silver nitrate; M: molecular mass standard (PageRuler  
760 low range unstained protein ladder); lanes 1 and 2: active fractions D7 and D8 from peak P3,  
761 respectively. (B) unstained gel, overlaid with *Hrr. chaoviator* ETR14 cells inoculated in SW  
762 soft agar; M: molecular mass standard (colored marker low range rainbow molecular weight);  
763 lane 1: purified fraction P3 from Phenyl-Sepharose. The lighter areas represent the archaea-  
764 free zones, where the activity of the halocins has inhibited the growth of *Hrr. chaoviator*  
765 ETR14. (C) Analysis of the MonoQ active fractions; Tris-tricine SDS-PAGE stained with  
766 silver nitrate; M: molecular mass standard (Ultra low range marker); lanes 1 and 2: active  
767 fractions E6 and E7 respectively. Arrows point the 8 kDa- and 14-kDa halocin bands.

768 Fig. 3. Amino-acid sequence analysis of the 8 kDa and 14 kDa halocins bands purified from  
769 *Hbt. salinarum* ETD5 culture supernatant. Localization of the peptides obtained by Edman  
770 degradation (white boxes) and LC/MS-MS of the chymotrypsin digests (grey boxes) in the  
771 amino acid sequences of (A) the HalS8 precursor and (B) the Mn-SOD from *Hbt. salinarum*  
772 NRC-1 (UniProt entry n° P09737). Vertical lines indicate proteolytic processings of the



773 halocin precursors: (A) N-terminal processing (black double arrow) common to this study and  
774 [20] and C-terminal processing (dotted line) hypothesized by [20]; the residue in bold has not  
775 been identified in Edman sequencing; stars point the eight cysteines. (B) Mn-SOD processing  
776 (black double arrow) hypothesized in this study. Residues in bold point the amino acids T, H  
777 and S, G in the Mn-SOD sequence that differ from those arising from HalS14 Edman  
778 sequencing (Q, X and I, I), and the amino acid in the Mn-SOD sequence (A) which differs  
779 from that arising from the peptide sequence obtained by LC-MS/MS (V). Stars point the  
780 manganese binding sites.

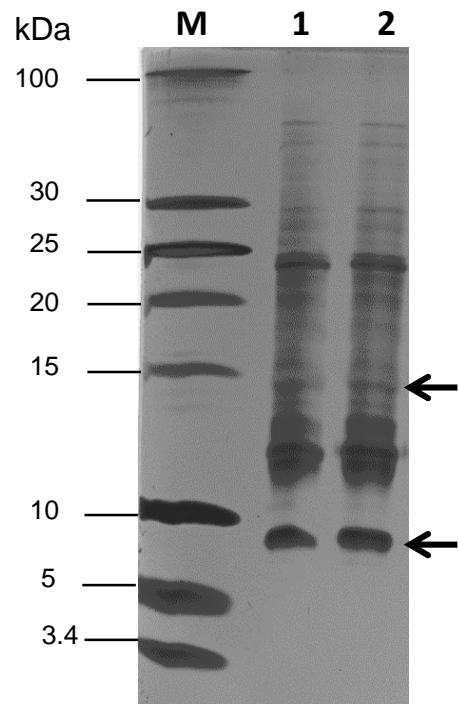
781 Fig. 4. Analysis of undigested 8 kDa halocin band by LC-MS. (A) Extracted ion  
782 chromatogram of the  $[M+11H]^{11+}$  species ( $m/z$  782) showing an elution in the 7–10 min  
783 range. (B) Mass spectrum (peaks shown with diamonds). (C) Enlargement of the mass  
784 spectrum showing the 11+ species. The  $m/z$  indicated correspond to monoisotopic values.

# Figure 1

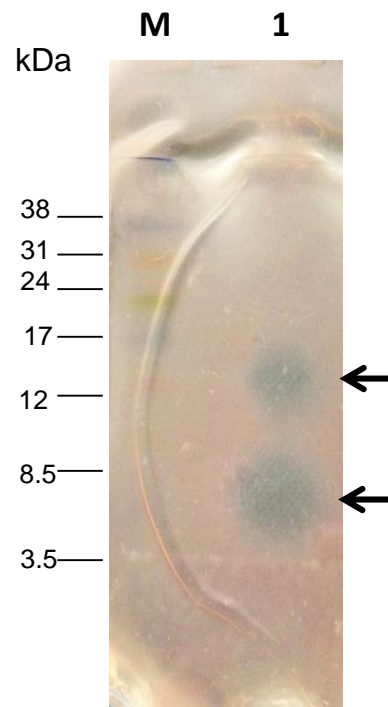
	1						60
Strain S8a	MSDKDSINRR	NVLRKIGGIG	VASAVGFSGI	ASGESLSDDE	KQDVIDTIYK	SQRVEQIKKK	
<i>Halobacterium</i> sp. GN101	MSDKDSINRR	NVLRKIGGIG	VASAVGFSGI	ASGESLSDDE	KQDVIDTIYK	SQRVEQIKKK	
<i>Hbt. salinarum</i> ETD5	MSDKDSINRR	NVLRKIGGIG	VASAVGFSGI	ASGESLSDDE	KQDVIDTIYK	SQRVEQIKKK	
	61						120
Strain S8a	FGGVNIEPKK	VQSVTTNQSG	DLVTAKLSVS	DGDLVYSSVK	DTTVIVQFDR	SASEIGESWP	
<i>Halobacterium</i> sp. GN101	FGGVNIEPKK	VQSVTTNQSG	DLVTAKLSVS	DGDLVYSSVK	DTTVIVQFDR	SASEIGESWP	
<i>Hbt. salinarum</i> ETD5	FGGVNIEPKK	VQSVTTNQSG	DLVTAKLSVS	DGDLVYSSVK	DTTVIVQFDR	SASEIGESWP	
	121						180
Strain S8a	KNTEAFIKST	SSGVDLLRTA	TDEEIKDVTE	GVNTSEIESA	DAVNIFIDPE	SQTYMEKYD	
<i>Halobacterium</i> sp. GN101	KNTEAFIKST	SSGVDLLRTA	TDEEIKDVTE	GVNTSEIESA	DAVNIFIDPE	SQTYMEKYD	
<i>Hbt. salinarum</i> ETD5	KNTEAFIKST	SSGVDLLRTA	TDEEIKDVTE	GVNTSEIESA	DAVNIFIDPE	SQTYMEKYD	
	181						240
Strain S8a	FNNKIVLEMFE	LATGGTSSGK	ISPTREDQNH	EYNVREHKVF	NSEKQNIQLQ	<u>SDCNINSNTA</u>	
<i>Halobacterium</i> sp. GN101	FNNKIVLEMFE	LATGGTSSGK	ISPTREDQNH	EYNVREHKVF	NSEKQNIQLQ	<u>SDCNINSNTA</u>	
<i>Hbt. salinarum</i> ETD5	FNNKIVLEMFE	LATGGTSSGK	ISPTREDQNH	EYNVREHKVF	NSEKQNIQLQ	<b>SDCNINSNTA</b>	
						*	
	241						300
Strain S8a	ADVILCFNQV	GSCALCSPTL	VGGPVPTVAC	LLVVCFGTPN	AVSAILEEVD	NSCFNLIKDV	
<i>Halobacterium</i> sp. GN101	ADVILCFNQV	GSCALCSPTL	VGGPVPTVAC	LLVVCFGTPN	AVSAILEEVD	NSCFNLIKDV	
<i>Hbt. salinarum</i> ETD5	<b>ADVILCFNQV</b>	<b>GSCALCSPTL</b>	<b>VGGPVPTVAC</b>	<b>LLVVCFGTPN</b>	<b>AVSAILEEVD</b>	<b>NSCFNLIKDV</b>	
	*	*	*	*	*	*	
	301	311					
Strain S8a	ISCWDEWTSF	W					
<i>Halobacterium</i> sp. GN101	ISCWDEWTSF	W					
<i>Hbt. salinarum</i> ETD5	<b>ISCWDEWTSF</b>	<b>W</b>					
	*						

**Figure 2**

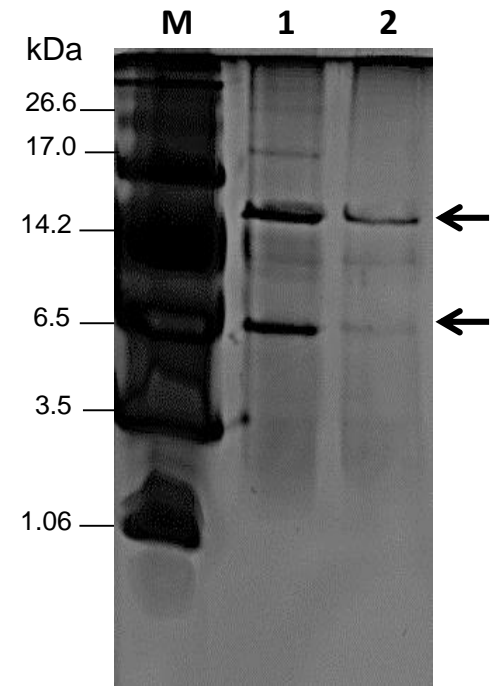
**A**



**B**



**C**



**Figure 3**

**A**

MSDKDSINRR	NVLRKIGGIG	VASAVGFSGL	ASGESLSDDE	KQDVIDTIYK	SQRVEQIKKK
FGGVNIEPKK	VQSVTTNQSG	DLVTAKLSVS	DGDLVYSSVK	DTTVIVQFDR	SASEIGESWP
KNTEAFIKST	SSGVDLLRTA	TDEEIKDVTE	GVNTSEIESA	DAVNIFIDPE	SQTYIMEKYD
FNNKVLEMFE	LATGGTSSGK	ISPTREDQNH	EYNVREHKVF	NSEKQNIQLQ	SDCNIN <b>S</b> NTA
ADVILCFNQV	GSCALCSPTL	VGGPVPTVAC	LLVVCFGTPN	AVSAILEEVD	NSCFNLIKDV
ISCWDEWTSF	W				

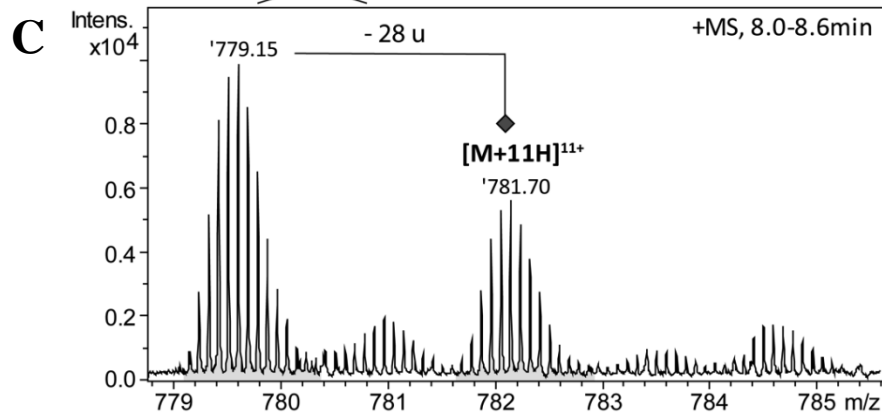
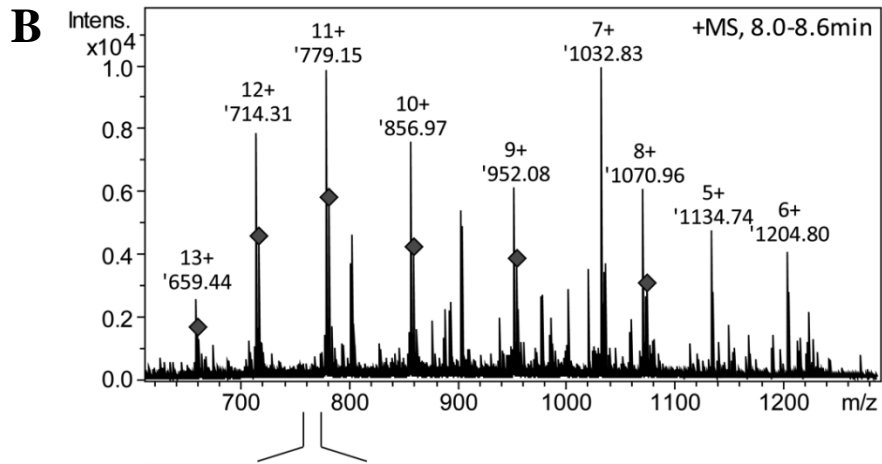
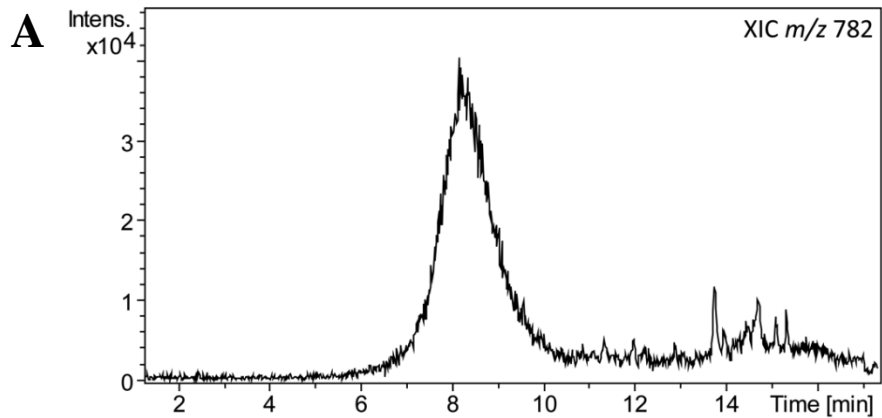
\* \* \* \* \*

**B**

MSEYELPPLP	YDYDALEPHI	SEQVLTWHHD	THHQGYVNGW	NDAEETLAEN	RETGDHASTA
GALGDV <b>THNG</b>	<b>SG</b> HILHTLFW	QSMSPAGGDE	PSGAL <b>ADRIA</b>	ADFGSYENWR	AEFEAAASAA
SGWALLVYDS	HSNTLRNVAV	DNHDEGALWG	SHPILAI <b>DVW</b>	EHSYYYDYGP	DRGSEFVDAFF
EVVDWDEPTE	RF <b>EQAAERFE</b>				

\* \* \* \* \*

**Figure 4**



**Table 1** Halophilic and non-halophilic strains used in this study.

Strain	Origin	Reference
<i>Haloarcula argentinensis</i> DSM 12282	Salinas Chica, Argentina	[22]
<i>Haloarcula hispanica</i> DSM 4426	Solar saltern, Spain	[23]
<i>Haloarcula japonica</i> DSM 6131	Solar saltern sediment, Japan	[24]
<i>Haloarcula</i> sp. SWI5	Solar saltern, Ichekaben, Algeria	[25]
<i>Halobacterium salinarum</i> DSM 3754	Salted cowhide	[26]
<i>Halobacterium salinarum</i> JCM 11081	Unknown	[27]
<i>Halobacterium salinarum</i> ETD5 <sup>a</sup>	Solar saltern, Sfax, Tunisia	[8]
<i>Halobacterium salinarum</i> ETD8	Solar saltern, Sfax, Tunisia	[8]
<i>Haloferax mediterranei</i> DSM 1411	Solar saltern, Alicante, Spain	[16]
<i>Haloferax volcanii</i> DSM 3757	Dead Sea, Israel, Jordan, Palestine	[28]
<i>Halorubrum chaoviator</i> ETD3	Solar saltern, Sfax, Tunisia	[8]
<i>Halorubrum chaoviator</i> ETR14	Solar saltern, Sfax, Tunisia	[8]
<i>Halorubrum saccharovororum</i> DSM 1137	Solar saltern, San Francisco, California	[29]
<i>Haloterrigena turkmenica</i> DSM 5511	Salt ground, Turkmenistan	[30]
<i>Natrinema altunense</i> JCM 12890	Salt lake Ayakekum, Xinjiang, China	[31]
<i>Natrinema gari</i> JCM 14663	Anchovy fish sauce, Thailand	[32]
<i>Natrinema pellirubrum</i> DSM 15624	Salted hide	[33]
<i>Halomonas sabkhae</i> DSM 19122	Sebkha Ezzemoul, Algeria	[34]
<i>Lentibacillus halophilis</i> DSM 19123	Fish sauce, Thailand	[35]
<i>Nesterenkonia halophila</i> DSM 16378	Ground, Xinjiang, China	[36]
<i>Oceanobacillus kapialis</i> DSM 23158	Fermented shrimp, Thailand	[37]
<i>Salinibacter ruber</i> DSM 13855	Solar saltern, Spain	[38]
<i>Salinicoccus siamensis</i> DSM 21576	Fermented shrimp, Thailand	[39]
<i>Escherichia coli</i> CIP 53.126	Faeces	[40]
<i>Kocuria rhizophila</i> CIP 53.45	Soil	[41]
<i>Pseudomonas aeruginosa</i> CIP 76.110	Blood	[42]
<i>Staphylococcus aureus</i> CIP 4.83	Human lesion	[43]
<i>Eurotium rubrum</i> LCP 6453	Spoiled almonds	[44]
<i>Penicillium corylophilum</i> LCP 5424	Soil	[45]

a

Producer of the antimicrobial peptides described in this study.

**Table 2** Activity spectrum of the *Hbt. salinarum* ETD5 supernatant against halophilic archaea, halophilic and non-halophilic bacteria, and halophilic fungi.

Target strains	Antimicrobial activity
<b>Halophilic archaea</b>	
<i>Haloarcula argentinensis</i> DSM 12282	+
<i>Haloarcula hispanica</i> DSM 4426	++
<i>Haloarcula japonica</i> DSM 6131	-
<i>Haloarcula</i> sp. SW15	+
<i>Halobacterium salinarum</i> DSM 3754	+
<i>Halobacterium salinarum</i> NRC1 JCM 11081	+++
<i>Haloferax mediterranei</i> DSM 1411	-
<i>Haloferax volcanii</i> DSM 3757	-
<i>Halorubrum chaoviator</i> ETR14	+++
<i>Halorubrum saccharovororum</i> DSM 1137	-
<i>Haloterrigena turkmenica</i> DSM 5511	++
<i>Natrinema altunense</i> JCM 12890	-
<i>Natrinema gari</i> JCM 14663	-
<i>Natrinema pellirubrum</i> DSM 15624	++
<b>Halophilic bacteria</b>	
<i>Halomonas sabkhae</i> DSM 19122	-
<i>Lentibacillus halophilis</i> DSM 19123	+
<i>Nesterenkonia halophila</i> DSM 16378	-
<i>Oceanobacillus kapialis</i> DSM 23158	-
<i>Salinibacter ruber</i> DSM 13855	+
<i>Salinicoccus siamensis</i> DSM 21576	-
<b>Non-halophilic bacteria</b>	
<i>Escherichia coli</i> CIP 53.126	-
<i>Kocuria rhizophila</i> CIP 53.45	-
<i>Pseudomonas aeuruginosa</i> CIP 76.110	+
<i>Staphylococcus aureus</i> CIP 4.83	-
<b>Halophilic fungi</b>	
<i>Eurotium rubrum</i> LCP 6453	-
<i>Penicillium coryphilum</i> LCP 5424	-

<sup>a</sup> Antimicrobial activity is expressed as inhibition halos (IH) in mm; +: IH ≤10; ++: 10 <IH ≤ 20; +++: IH >20; -: no inhibition zone.

**Table 3** Effects of treatments with proteolytic enzymes, variations of NaCl concentration and detergents on the antimicrobial activity of the *Hbt. salinarum* ETD5 crude supernatant on *Hrr.chaoviator* ETR14.

Treatment	Inhibition halos <sup>a</sup> (mm)	
<b>Proteases</b>		
Untreated supernatant	32 ± 0.8	
20 ng μL <sup>-1</sup> Chymotrypsin	0	
20 ng μL <sup>-1</sup> Thermolysin	0	
20 ng μL <sup>-1</sup> Endoproteinase GluC	0	
<b>NaCl concentration</b>		
Untreated supernatant	32 ± 0.8	
0 M NaCl <sup>b</sup>	18 ± 1.6	
0.5 M NaCl	18 ± 0.8	
1 M NaCl	20 ± 1.6	
1.5 M NaCl	24 ± 0.8	
2 M NaCl	25 ± 1.6	
2.5 M NaCl	28 ± 1.6	
3 M NaCl	30 ± 0.8	
3.5 M NaCl	30 ± 1.6	
4 M NaCl	27 ± 1.6	
<b>Detergents</b>		
Proportion of detergent	Detergent	
	Tween-20	Triton X-100
0‰	32 ± 0.8	32 ± 0.8
1‰	12 ± 1.6	13 ± 1.6
2‰	12 ± 0.0	14 ± 0.0
3‰	12 ± 0.8	14 ± 0.8
4‰	15 ± 0.8	15 ± 1.6
5‰	13 ± 1.6	15 ± 0.8
6‰	14 ± 1.6	12 ± 0.8
7‰	13 ± 0.8	11 ± 0.8
8‰	16 ± 1.6	12 ± 1.6
9‰	13 ± 0.8	12 ± 0.8
10‰	12 ± 1.6	13 ± 0.8

<sup>a</sup> The antimicrobial assays were carried out in triplicate. The antimicrobial activity is expressed as the average of the three diameters of inhibition halos measured in mm with standard deviations.

<sup>b</sup> Supernatant dialyzed against Milli-Q<sup>®</sup> water.