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Growth Characteristics and Halocin Production by a New Isolate, *Haloferax volcanii* KPS1 from Kovalam Solar Saltern (India)

[1]P. Kavitha, [2]A.P. Lipton, [1]A.R. Sarika and [1]M.S. Aishwarya
[1]Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Tamil Nadu, India
[2]Central Marine Fisheries Research Institute, Vizhinjam Research Centre of CMFRI, 695521-Vizhinjam, Kerala, India

Abstract: An archeon *Haloferax volcanii* KPS1 was isolated from Kovalam saltern, Kanyakumari, India. It produced halocin which was active against gram positive and gram negative bacteria viz., *Bacillus subtilis*, *Streptococcus mutans*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, respectively. The halobacteria *H. sodomens* was inhibited to the maximum by the halocin KPS1 of the *Haloferax volcanii* KPS1. The growth and halocin production was maximized when the organism was grown at a temperature of 40°C at the pH of 7.0 and when supplemented with 25% w/v NaCl to the halophilic broth medium. The results of the stability studies indicated that the halocin KPS1 became thermolabile at the temperature >80°C and was stable over a wide range of pH from 3.0-9.0. A loss of activity of halocin KPS1 was detected when treated with proteolytic enzymes like proteinase K and trypsin indicating the proteinaceous nature of the antibacterial compound. The halocin produced by *Haloferax volcanii* KPS1 would provide a lead for developing antimicrobial drugs.

Key words: *Haloferax volcanii* KPS1, halocin KPS1, halophilic agar, culture conditions, enzymes, India

INTRODUCTION

Extremely halophilic archaea belong to a single euryarchaeotal order (Halobacteriales) that inhabits various hyper saline environments (3-5 M) such as salt lakes, salt ponds and marine salterns. Previous molecular ecology studies showed that archaeal halophiles dominate these ecosystems. 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. 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. 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 inhibit organisms closely related to the producer. The formation of bacteriocins by a member of the Halobacteriaceae, *Haloferax mediterranei* was first described in 1982 by Rodriguez-Valera *et al.* (1982, 1983). During the last decade, such protein antibiotics have been studied widely due to their potential as preserving agents in the food industry, controlling agents for infectious bacteria etc. It has been documented that halocin production is an almost universal feature among the halobacteria (Meseguer *et al*., 1986; Shand *et al*., 1999; Torreblanca *et al*., 1994).

Based on antagonism studies, hundreds of different types have been found to exist. Despite their abundance, only a handful of halocins have been characterized at the protein level (halocins H4, H6 and R1) (Meseguer and Rodriguez-Valera, 1985; Rdest and Sturm, 1987) and only one Halocin (H4) has been characterized at both the gene and mRNA transcript levels. A halophilic archae KPS1 isolate from solar saltern with high antibacterial potential was isolated was characterized in the present study. The growth characteristics of this specie and the halocin

Corresponding Author: P. Kavitha, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Tamil Nadu, India

production at different culture conditions were determined so as to maximize the yield of the antibacterial protein.

MATERIALS AND METHODS

Environmental sample and isolation of *Haloferax volcanii* KPS1: The water samples were collected from crystallizer ponds of Kovalam salt pan (longitude 77.2 and latitude 8.3) during the month of January, 2008. About 10 mL of the sample was enriched in 40 mL Halophilic broth (Hi-Media M590) and incubated for 7 days at 40°C. The *Haloferax volcanii* KPS1 was isolated by serial dilution and plating of the enriched broth in Halophilic agar (Hi-Media M590).

Screening for halocin production and characterization of halobacterium: The isolated strain of *Haloferax volcanii* KPS1 was screened for its antagonistic potential against the indicator strains using the overlay technique (Cooper and James, 1984).

The indicator strains used include the pure cultures of *Halobacterium sodomense* obtained from the lab (CMST) stock and *Staphylococcus aureus* MTCC 916, *Escherichia coli* MTCC 1671, *Bacillus subtilis* MTCC 1134, *Streptococcus mutans* MTCC 896, *Pseudomonas aeruginosa* MTCC 6538 obtained Microbial Type Culture Collection (MTCC), Chandigarh, India. The strain which showed antagonistic activity against indicator strains was selected and characterized as *Haloferax volcanii* KPS1 based on the cell morphology, biochemical identification and 16S rRNA sequencing. The halocin produced by *Haloferax volcanii* KPS1 was designated as halocin KPS1.

Assay of halocin activity: The halocinogenic activity of *Haloferax volcanii* KPS1 was monitored against *H. sodomense* and with other gram negative and positive strains using agar well-diffusion method (Geis *et al*., 1983). The cell-free culture supernatant of *Haloferax volcanii* KPS1 was diluted 2-fold and 50 µL was poured on to 6 mm well made on to the lawn of indicator strains. The highest dilution producing visible inhibition per mL cell-free supernatant was considered as containing one arbitrary unit per mL (AU mL^{-1}).

Growth and physiological characteristics of *Haloferax volcanii* KPS1: The growth and physiology of *Haloferax volcanii* KPS1 was monitored in Halophilic broth incubated at 40°C for 7 days while samples were withdrawn every 24 h interval to detect the optical density (600 nm) and cfu mL^{-1}. The inoculum size of 0.1 mL (approximate 10^8 cfu mL^{-1}) was aseptically transferred to 4.0 mL of halophilic broth for each test. The effect of different temperatures viz., 10, 20, 30, 40 and 50°C, pH viz., 4, 5, 7, 9 and 11 and salinity viz., 0, 3, 8, 15, 20, 25 and 32% NaCl on the growth of the *Haloferax volcanii* KPS1 was determined by incubating the inoculated broth at the respective conditions for 7 days and spectrophotometric determination at every 24 h interval.

Effect of culture conditions on halocin KPS1 production: To determine the optimum conditions (viz., incubation period, temperature, pH and salinity) of halocin production, the cell-free supernatant of *Haloferax volcanii* KPS1 from the experimental broth was collected at every 24 h interval and assayed as described before.

Stability of halocin KPS1 at different temperature and pH: The stability of the halocin KPS1 to heat was determined by exposing the cell free supernatant to 50, 60, 70, 80, 90, 100 and 121°C for 15 min and determining the halocin activity in terms of AU. The stability to pH was detected by adjusting the cell-free supernatant to different pH range from 3-9 with sterile 1 N NaOH and 1 N HCl and incubation for 2 h. The results in terms of AU mL^{-1} were recorded by halocin assay.

Stability to proteolytic enzymes: The stability of halocin KPS1 to proteolytic enzymes was determined by incubating the cell free supernatant of *Haloferax volcanii* KPS1 with proteinases K (100 µg mL^{-1} and 1 mg mL^{-1}) and trypsin (100 µg mL^{-1} and 1 mg mL^{-1}) for 60 min at room temperature (27±2°C).

RESULTS AND DISCUSSION

Isolation and characterization of *Haloferax volcanii* KPS1: The *Haloferax volcanii* KPS1 isolated from saltern using halophilic agar as the selective medium was gram negative, aerobic rods. The strain was catalase and oxidase positive and did not produce capsules or spores. The whole sequence of 16S rRNA of the *Haloferax volcanii* KPS1 was aligned with the sequence of other *Halobacterium* sp. and some other archaebacteria using BLAST2 search. The complete 16S rDNA sequence of the *Haloferax volcanii* KPS1 was compared with type strain of Halobacterium and other archaebacteria from the similarity matrix, calculated by number of base differences. Based on the biochemical tests and the 16S rRNA sequencing, the isolated halocin producing halobacteria was characterized and designated as *Haloferax volcanii* KPS1 and the halocin produced

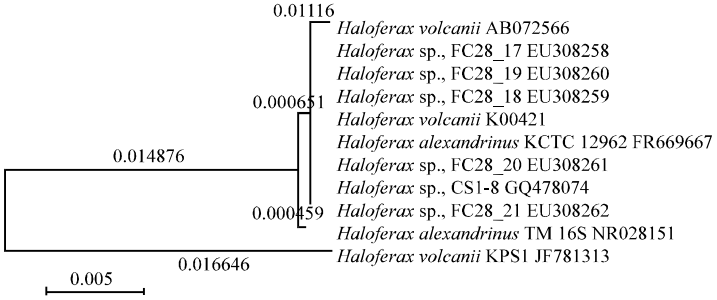

Fig. 1: Phylogenetic tree constructed using MEGA 5.03 showing the relationship of *Haloferax volcanii* KPS1 with 16S rRNA of other archaebacteria

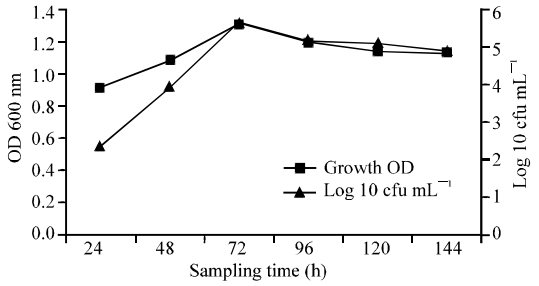

Fig. 2: Growth pattern of *Haloferax volcanii* KPS

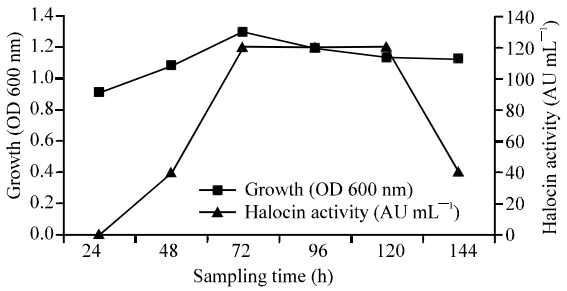

Fig. 3: Halocin KPS1 level in relation to the growth of *Haloferax volcanii* KPS1

was designated as halocin KPS1. Distance matrix tree using neighbour joining method was constructed using MEGA 5.03 as shown in Fig. 1. The *16S* rRNA gene sequence of *Haloferax volcanii* KPS1 was deposited in the GenBank and was given the Accession No. JF781313.

Growth pattern of *Haloferax volcanii* KPS1: The growth (OD 600 nm) of the strain S1 increased drastically between 24-72 h after inoculation entered the stationary phase after 72 h and declined after 120 h. The results of the growth experiments are shown in the Fig. 2. The growth was maximized when the culture broth was incubated at a temperature of 40°C and when the initial medium pH was adjusted to 7.0. In addition, the supplementation of 25% w/v NaCl to Halophilic agar was noticed to be ideal for the growth of *Haloferax volcanii* KPS1. The growth characteristics of *Haloferax volcanii* KPS1 was supported by the findings made earlier with an uncharacterized haloarcheon strain S8a isolated from Great Salt lake (Price and Shand, 2000) in which the growth optimum was noticed at the temperature of 40°C. Further, the high salt requirements of the isolated strains indicated that these organisms belong to the extreme halophile group (Norton, 1992).

Pattern of halocin production by *Haloferax volcanii* KPS1: Batch culture growth experiments (OD 600 nm) with strain KPS1 showed that maximum halocin activity occurred in early stationary phase (72 h) persisted at these levels for 120 h and decreased thereafter. Thus, it could be inferred that *Haloferax volcanii* KPS1 isolate showed growth stability and halocin production at the stationary phase. The studies conducted earlier on a few halocins revealed that most of them were expressed initially at transition to stationary phase (Cheung *et al*., 1997; Price and Shand, 2000; Rdest and Sturm, 1987; O'Connor and Shand, 2002). It had been reported that for the other bacteriocins also the activity in broth cultures reached maximum only after the exponential growth had ceased (Jose *et al*., 1998). The observations made earlier with halocin S8a and halocin C8 are in tune with the results of the present investigation (Price and Shand, 2000; Li *et al*., 2003). The halocin activity was detected (40 AU mL^{-1}) at the late exponential phase which reached the maximum (120 AU mL^{-1}) at the stationary phase of growth (Fig. 3).

Antibacterial spectrum of halocin KPS1 produced by *Haloferax volcanii* KPS1: The inhibitory activity pattern of halocin KPS1 against halobacteria and other pathogens are shown in Table 1. A broad spectrum of activity was noticed with the halocin in that it inhibited both the gram positive and gram negative pathogenic strains. While the

Table 1: Antibacterial activity of halocin KPS1 produced by *Haloferax volcanii* KPS1

Bacterial strains	Overlay method	Well-diffusion assay (AU mL^{-1}*)
Halobacterium sodomense S2	++	120
Escherichia coli MTCC 1671	+	40
Streptococcus mutans MTCC 896	+	80
Pseudomonas aeruginosa MTCC 6538	+	40
Bacillus subtilis MTCC 1134	++	120
Staphylococcus aureus MTCC 916	+	80

*AU mL^{-1} = Arbitrary Units mL^{-1}; ++ = High activity; + = Moderate activity

halocin inhibited *Halobacteria* sp., to the maximum, it was potent enough to restrain the growth of other unrelated pathogenic strains such as *E. coli* MTCC 1671, *S. mutans*, MTCC 896, *P. aeruginosa*, MTCC 6538, *B. subtilis* MTCC 1134 and *S. aureus*. MTCC916 similar observations in par with this had been made by Chen *et al.* (2010) in which three strains of the halophilic bacteria isolated from solar saltern in China inhibited *E. coli* and *P. fluorescens*. In contrast, the halocin S8 and C8 observed to have a narrow inhibitory spectrum and inhibited only related *Halobacteria* sp. (Price and Shand, 2000; Li *et al*., 2003).

Effect of culture conditions on halocin KPS1 production: The temperature of incubation exerted a profound influence on halocin KPS1 production by *Haloferax volcanii* KPS1 as was evidenced from observations made in the present study (Table 2).

The halocin KPS1 production maximized when incubated at 40°C at a pH of 7.0 upon supplementation with 25% w/v NaCl. It could be noted from Table 2 that the halocin activity increased with an increase of temperature up to 40°C and subsequently decreased with further rise in temperature. The highest activity of the halocin was detected at the pH of 7.0, though only a slight reduction in the activity was noticed at the pH of 9.0. This suggested that halocin production optimized between pH 7-8 for *Haloferax volcanii* KPS1.

The maximum activity detected at 25% NaCl indicated the fact that the halocin production was salt-dependent. As reported before (Enache *et al*., 2007), the Halobacteria survive in the saltern with pH of 8.0 at a salt concentration of 161 g L^{-1} present in the surface water. It was also suggested that the halocin production provided mechanisms by which an individual species avoid competition with other species requiring similar environmental conditions. These earlier findings support the present observations of the halocin production at a high salt concentration (25% w/v) in the *in vitro* conditions. Halocins already characterized differ in their ionic stability. A few halocins viz., R1, H6, Ha1 and S8 are salt-independent (Price and Shand, 2000; Rdest and Sturm, 1987; Torreblanca *et al*., 1989).

Table 2: Effect of culture conditions on growth and halocin KPS1 production by *Haloferax volcanii* KPS1

Growth parameters	Growth (OD 600 nm)	Halocin production (AU mL^{-1})
Temperature (°C)		
10	0.068	-
20	0.071	-
30	0.180	-
40	1.310	120
50	0.720	80
pH		
4	0.510	-
5	0.590	-
7	1.890	160
9	1.750	120
11	0.470	-
Salinity (NaCl%)		
0	0.006	-
3	0.040	-
8	0.091	-
15	0.380	40
20	0.720	80
25	1.260	120
32	0.810	80

Table 3: Effect of heat treatment and pH on the inhibitory activity of halocin KPS1

Treatments	Activity (AU)
Untreated	120
Heat (°C)	
50	120
60	120
70	120
80	80
90	-
100	-
121	-
pH	
3	120
4	120
5	120
6	120
7	120
8	120
9	80

Stability of halocin KPS1 to pH and heat treatment: The halocin exhibited consistent stability over the wide range of pH (3.0-9.0) at room temperature with a reduction in activity at a high alkaline pH (Table 3). The protein was thermolabile at temperature >80°C which was in accordance with the observation made for the bacteriocin produced by a halophilic Archeon Sec 7a (Pasic *et al*., 2008). This observation is in contrary to that of halocin H6 produced by *Haloferax gibbonsii* which was thermostable up to boiling temperatures (Torreblanca *et al*., 1989).

Stability to proteases: The halocin activity was lost when incubated with 1 mg mL^{-1} of Proteinases K and Trypsin for 10 min. However, the protein remained stable in the presence of 100 μg mL^{-1} Trypsin when observed after 10 min; further increase in the incubation period led to the

complete loss of activity. The loss of activity in the presence of proteases could be attributed to the degradation of the peptide by the proteolytic enzymes and thus, confirming the proteinaceous nature of the antimicrobial compound as suggested by Shand and Leyva (2007).

CONCLUSION

The studies made earlier evidences that the vast majority of antimicrobial products have come from non-extreme, terrestrial microorganism. However while these micro-organisms continue to be studied extensively, the rate of discovery of novel metabolites from terrestrial micro-organisms is decreasing. Discovery and identification of natural antimicrobial products from new sources viz., the extreme environments therefore plays an important role in the uncovering of novel drug leads and drug development process (Singh and Macdonald, 2010). The broad spectrum of activity and stability to pH and heat observed with halocin S1 would be beneficial in possible development of drugs with this antimicrobial peptide.

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REFERENCES

Chen, J., L. Zhang, G.C. Paoli, C. Shi, S.I. Tu and X. Shi, 2010. A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. Int. J. Food Microbiol., 137: 168-174.

Cheung, J., K.J. Danna, E.M. O'Connor, L.B. Price and R.F. Shand, 1997. Isolation, sequence and expression of the gene encoding halocin H4, a bacteriocin from the halophilic archaeon *Haloferax mediterranei* R4. J. Bacteriol., 179: 548-551.

Cooper, P.C. and R. James, 1984. Two new E colicins, E8 and E9, produced by a strain of *Escherichia coli*. J. Gen. Microbiol., 130: 209-215.

Enache, M., T. Itoh, T. Fukushima, R. Usami, L. Dumitru and M. Kamekura, 2007. Phylogenetic relationships within the family Halobacteriaceae inferred from rpoB gene and protein sequences. Int. J. Syst. Evol. Microbiol., 57: 2289-2295.

Geis, A., J. Singh and M. Tueber, 1983. Potential of lactic streptococci to produce bacteriocin. Applied Environ Microbiol., 45: 205-211.

Jose, B.F., L. Jose, R. Barba and R.J. Diaz, 1998. Purification and genetic characterization of the enterocin I from *Enterococcus faecium* 6 T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocin. Applied Environ. Microbiol., 64: 4883-4890.

Li, Y., H. Xiang, J. Liu, M. Zhou and H. Tan, 2003. Purification and biological characterization of Halocin C8, a novel peptide antibiotic from *Halobactrium* strain AS 7092. Extremophiles, 7: 401-407.

Meseguer, I. and F. Rodriguez-Valera, 1985. Production and purification of halocin H4. FEMS Microbiol. Lett., 28: 177-182.

Meseguer, I., F. Rodriguez-Valera and A. Ventosa, 1986. Antagonistic interactions among halobacteria due to halocin production. FEMS Microbiol. Lett., 36: 177-182.

Norton, C., 1992. Rediscovering the ecology of halobacteria. ASM News, 58: 363-367.

O'Connor, E.M. and R.F. Shand, 2002. Halocins and sulfolobicins: The emerging story of archaeal protein and peptide antibiotics. J. Ind. Microbiol. Biotechnol., 28: 23-31.

Pasic, L., B.H. Velikonja and N.P. Ulrih, 2008. Optimization of the culture conditions for the production of a bacteriocin from halophilic archaeon Sech7a. Prep. Biochem. Biotechnol., 38: 229-245.

Price, L.B. and R.F. Shand, 2000. Halocin S8: A 36-amino-acid microhalocin from the *Haloarchaeal* strain S8a. J. Bacteriol., 182: 4951-4958.

Rdest, U. and M. Sturm, 1987. Bacteriocins from Halobacteria. In: Protein Purification: Micro to Macro, Burgess, R. (Ed.). John Wiley and Sons Canada Limited, New York, USA., ISBN-13: 9780471607243, pp: 271-278.

Rodriguez-Valera, F., G. Juez and D.J. Kushner, 1982. Halocins: Salt dependent bacteriocins produced by extremely halophilic rods. Can. J. Microbiol., 28: 151-154.

Rodriguez-Valera, F., G. Juez and D.J. Kushner, 1983. *Halobacterium mediterranei* sp. nov., a new carbohydrate-utilizing extreme halophile. Syst. Applied Microbiol., 4: 369-381.

Shand, R.F. and K.J. Leyva, 2007. Antibiotics from Domain Archae: Halocins and Sulfobiocins. In: Bacteriocins: Ecology and Evolution, Riley, M.A. and M.A. Chavan (Eds.). Springer, New York, USA., ISBN-13: 9783540366034, pp: 93-109.

Shand, R.F., L.B. Price and E.M. O'Connor, 1999. Halocins: Protein Antibiotics from Hypersaline Environments. In: Microbiology and Biogeochemistry of Hypersaline Environments, Oren, A. (Ed.). CRC Press, Boca Raton, FL., USA., pp: 295-306.

Singh, B.K. and C.A. Macdonald, 2010. Drug discovery from uncultivable microorganisms. Drug Discovery Today, 15: 792-799.

Torreblanca, M., I. Meseguer and A. Ventosa, 1994. Production of halocin is a practically universal feature of archaeal halophilic rods. Lett. Applied Microbiol., 19: 201-205.

Torreblanca, M., I. Meseguer and F. Rodriguez-Valera, 1989. Halocin H6, a bacteriocin from *Haloferax gibbonsii*. J. General Microbiol., 135: 2661-2665.