Extremophiles
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Exploring the multiple biotechnological potential of halophilic microorganisms isolated from two Argentinean salterns

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Abstract The biodiversity and biotechnological potential of microbes from central Argentinean halophilic environments have been poorly explored. Salitral Negro and Colorada Grande salterns are neutral hypersaline basins exploded for NaCl extraction. As part of an ecological analysis of these environments, two bacterial and seven archaeal representatives were isolated, identified and examined for their biotechnological potential. The presence of hydrolases (proteases, amylases, lipases, cellulases and nucleases) and bioactive molecules (surfactants and antimicrobial compounds) was screened. While all the isolates exhibited at least one of the tested activities or biocompounds, the species belonging to *Haloarcula* genus were the most active, also producing antimicrobial compounds against their counterparts. In general, the biosurfactants were more effective against olive oil and aromatic compounds than detergents (SDS or Triton X-100). Our results demonstrate the broad spectrum of activities with biotechnological potential exhibited by the microorganisms inhabiting the Argentinean salterns and reinforce the importance of screening pristine extreme environments to discover interesting/novel bioactive molecules.

Keywords Halophilic microorganisms · Hydrolytic activities · Bioactive molecules · Biosurfactant

Introduction

The improvement of current industrial processes requires molecules that remain active and stable under extreme conditions of pH, temperature, ionic strength and/or limited solubility. Therefore, screening new sources for novel enzymes or byproducts is fundamental to extend the possibilities of Biotechnology. Extreme environments such as hypersaline water bodies containing salt concentrations nine to tenfold higher than that of sea water (30–35 vs. 3.5 % NaCl, respectively) are widely distributed around the world and have a diverse microbial population composed by halophilic organisms from the three domains of life. Since the last decade, microorganisms thriving in these environments have been explored as a source of novel compounds due to the ability of their macromolecules to remain active at ionic strengths higher than 2 M NaCl (low water activity), temperatures over 40 °C and, in some instances, pH values higher than 9 (Delgado-Garcia et al. 2012; Litchfield 2011; Margesin and Schinner 2001; Moreno et al. 2013; Oren 2010).

Halophilic proteins are rich in acidic amino acids, thus, they exhibit high density surface charges, which interact with chloride and sodium ions to form salt bridges. These features contribute to the solubility and structural stability of halophilic enzymes in low water activity environments, making them sought and appreciated for biocatalysis (Madern et al. 2000; Marhuenda-Egea and Bonete 2002; Richard

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et al. 2000). Enzymes such as glycoside hydrolases, lipases, proteases, and nucleases from halophiles have been analyzed as candidates for industrial applications in different fields (Delgado-Garcia et al. 2012; Litchfield 2011; Marhuenda-Egea and Bonete 2002; Moreno et al. 2013; Oren 2010) although the variety of hydrolytic profiles and the wide microbial diversity make worthy further and exhaustive screenings in hypersaline environments. Moreover, secondary metabolites produced by halophiles including biopolymers, surfactants, extracellular polysaccharides, bacteriorhodopsins and halocins have been the subject of a number of recent studies aiming to explore their potential biotechnological applications (Cameotra and Makkar 1998; Litchfield 2011; Oren 2010; Satpute et al. 2010).

Argentina exhibits extremely saline environments as diverse as the high altitude lakes in the North Andes or the hypersaline salterns in soil depressions of Patagonia. Their microbial diversity and biotechnological potential are being examined taking into account their interesting, unusual and extreme features. For instance, several UV-resistant microorganisms were isolated from the high altitude Andean saline lakes (Albarracín et al. 2012) and the adaptative response of microorganisms to the elevated arsenic concentration predominating in these waters is currently under study (Belfiore et al. 2013).

In this work we explored Salitral Negro and Colorada Grande salterns located in La Pampa province (Argentina). They are closed and neutral basins with salinities ranging from 30 to 38 % NaCl over the year. Since the climate of the region is semiarid, large thermal amplitudes are usual. Although these two environments are important working mines for NaCl extraction and production, their microbial diversity has not been examined so far. In this study, we isolated and identified several halophilic microorganisms from Salitral Negro and Colorada Grande salterns and explored their capacity to produce molecules with biotechnological potential.

Materials and methods

Chemicals

All the reagents used in this work were purchased in SIGMA-Aldrich (Bs As, Argentina) with the exception of yeast extract (YE) and agar which were from Oxoid (Hmpshire, England) and NaCl that was bought at J. T. Baker (PA, USA).

Isolation and identification of microorganisms

Water samples from Colorada Grande (38°15′S, 63°45′W) and Salitral Negro (38°43′01″S, 64°09′01″W) hypersaline ponds were inoculated into 25 % (w/v) SW medium (Antón et al. 2002) containing (g/L): 195 g NaCl, 34.6 g $MgCl_2{\cdot}6H_2O$, 49.5 g $MgSO_4{\cdot}7H_2O$, 1.25 g $CaCl_2{\cdot}2H_2O$, 5 g KCl, 0.25 g $NaHCO_3$, 0.625 g NaBr and 2.0 g YE. Additionally, these samples were inoculated into autoclaved water from each saltern to which 1 or 5 % (w/v) YE was added. Liquid cultures were incubated at 37 °C in an orbital shaker at 150 rpm for at least a week and then a sample was streaked onto agar plates (1.2 %, w/v) containing the same medium. Bacterial colonies were re-streaked 10 times to obtain pure cultures and used for DNA extraction according to Mutlu et al. (2008). To identify the isolated microorganisms, 16S ribosomal RNA genes were PCR-amplified and sequenced. PCR reactions were carried out with universal primers specific for conserved regions of SSU rRNA genes of *Archaea* (21F and 1492R) and *Bacteria* (27F and 1492R) (Delong 1992; Lane et al. 1985). Amplicons were gel-purified (kit Qiagen) according to the manufacturer's recommendations and sequenced at the INTA Castelar DNA Sequencing Service (Argentina). DNA sequences were analyzed against Public databases using the BLAST (Basic Local Alignment Search Tool) software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

For the following assays, the isolates were cultured in SW 25 % (w/v) liquid medium and incubated at 37 °C (except when indicated) in an orbital shaker at 150 rpm. The plates were incubated on a shelf at 37 °C.

Screening extracellular activities

The isolates were streaked onto the solid media co-polymerized with the corresponding substrate.

Protease activity

Protease activity was assayed on SW agar plates containing skim milk (0.8 %, w/v) or hemoglobin (0.4 %, w/v). Clear halos around the colonies were considered as evidence of proteolytic activity.

Amylase activity

The presence of extracellular amylase activity was determined using SW plates supplemented with soluble starch (1 %, w/v). After incubation, the plates were flooded with I_2–KI (0.3 and 0.6 %, w/v) solution. A clear zone around the colonies indicated hydrolysis of starch.

Lipolytic activity

The ability of the isolates to degrade different lipids was detected by the presence of yellow hydrolysis zones around

the colonies grown onto SW plates containing 1 % (w/v) olive oil and 0.1 % (w/v) phenol red. The color of the halo was produced by the decrease in pH due to the release of fatty acids from oil (Bhatnagar et al. 2005).

To determine the specificity of fatty acids cleavage, solid medium was co-polymerized with 1 % (v/v) Tween 20 or Tween 80 as source of saturated or unsaturated acids, respectively. Tween 20 and 80 are esters of lauric and oleic acids, respectively, allowing to test the enzyme substrate preference. A milky halo produced by precipitation of the fatty acid-calcium salt was considered as positive (Tirunarayanan and Lundbeck 1968).

Cellulolytic activity

The isolates were plated onto 1 % (w/v) carboxymethyl cellulose (CMC)-SW agar plates. After incubation, the plates were flooded with 0.15 % (w/v) Congo Red stain, washed with 1 M NaCl and acidified with 1 M HCl to intensify contrast (Gohel et al. 2014).

DNase activity

SW agar medium supplemented with 0.5 % (w/v) salmon sperm DNA was used for the detection of strains with DNase activity. After incubation, the plates were flooded with 1 M HCl solution. Clear halos around the colonies indicated activity.

Emulsification activity assay

All strains were grown to an optical density at 600 nm (OD_{600}) = 1 and centrifuged at 10,000×*g* 10 min to obtain cell-free medium as biosurfactant source. The emulsification activity was measured using olive oil or xylenes as substrate. Equal volumes of cell-free medium and substrate were vortexed at high speed for 2 min. The mixture was allowed to stand for 2 h before measurement. The emulsification activity was defined as the ratio between the height of the emulsion layer and the total height and expressed as percentage (Cooper and Goldenberg 1987). The emulsions were kept at room temperature and the stability was followed for 15 days.

An attempt to isolate the molecule/s involved in the emulsification activity was performed using acidic precipitation of the cell-free culture media. To this end, HCl was added to the cell-free medium to reach pH 2 and then incubated over night at 4 °C. After centrifugation at 17,000×*g* for 30 min, the pellet and supernatant were separated. The pellet was suspended in double-distilled water whereas the supernatant was spliced in two aliquots, one of which was neutralized with the addition of 10 N NaOH (Kebbouche-Gana et al. 2013).

To improve surfactant production, the isolates were grown under different conditions. Two different temperatures (30 and 55 °C) and salinities (2.5 and 5.0 M NaCl) were assayed. Casamino acids were used as carbon and nitrogen source instead of YE.

Halocin activity

All strains were tested against each other using the double layer method (Torreblanca et al. 1994). Cultures were grown to an OD_{600} = 1.0 and the plates were assembled as follows: SW top agar (0.7 %, w/v) containing the indicator strain (100 µl microbial culture/10 ml medium) was poured on the top of the same sterile solid medium. When solidified, 10 µl of a cell suspension of each of the strains was spotted onto these plates. They were incubated until a homogenous microbial lawn was developed. The presence of clear zones in the lawn around the culture drops indicated halocin activity.

Result and discussion

Extreme halophilic microorganisms grow in hypersaline environments where oligotrophic nutritional conditions, extreme temperature fluctuations and high levels of sunlight irradiation are usual parameters. To thrive into these low water activity environments, they have evolved to produce enzymes and metabolites that are functional in the predominating harsh conditions of their surroundings. As a consequence of this selective pressure, in addition to the stability at high salt concentration (30–35 %), their hydrolases are rather tolerant to high temperatures (50 °C or more) and functional in presence of organic solvents, conditions that make these molecules suitable for biotechnological applications in food processing, environmental bioremediation and biosynthetic processes (Delgado-Garcia et al. 2012; Litchfield 2011; Moreno et al. 2013; Oren 2010). In addition to hydrolases (proteases, amylases, lipases, nucleases), halophiles synthesize several secondary metabolites such as polymers, surfactants, bacteriorhodopsin and halocins that in the last years were subject of an increased interest due to their potential application in different processes (Litchfield 2011; Margesin and Schinner 2001; Oren 2010).

Isolation and identification of halophilic microorganisms

Water samples from Salitral Negro and Colorada Grande salterns were inoculated into different saline media to isolate culturable representatives. After several re-inoculations, isolated colonies showing distinct morphological characteristics (color, shape) were obtained. To perform the

Table 1 Halophilic strains isolated in this work

Isolate name	Domain	Accession number	Saltern	Culture medium	Identity (%)
C	*Archaea*	KP760841 (679 bp)	Colorada Grande	SW + 2 g/L YE	*Haloarcula argentinensis* (NR_112708); 100
G	*Archaea*	KP760844 (560 bp)	Colorada Grande	SW + 5 g/L YE	*Haloarcula japonica* (NR_112710); 100
V	*Archaea*	KP760848 (602 bp)	Colorada Grande	SW + 5 g/L YE	*Haloarcula vallismortis* (NR_112707); 98
F	*Archaea*	KP760843 (705 bp)	Colorada Grande	SW + 1 g/L YE	*Halorubrum tebenquichense* (HQ641750); 99
2	*Archaea*	KP760840 (684 bp)	Salitral Negro	SW + 5 g/L YE	*Halobacterium salinarum* (DQ465019); 99
E	*Archaea*	KP760842 (712 bp)	Colorada Grande	SW + 5 g/L YE	*Halobacterium* sp. (AB603514); 99
P	*Archaea*	KP760847 (703 bp)	Salitral Negro	SW + 5 g/L YE	*Halobacterium piscisalsi* (JX067388); 100
Kr	*Bacteria*	KP760846 (650 bp)	Colorada Grande	SW + 2 g/L YE	*Salinibacter ruber* strain M8 (FP565814); 100
Kb	*Bacteria*	KP760845 (667 bp)	Colorada Grande	SW + 2 g/L YE	*Salicola* sp. (EU931310); 99

Table 2 Hydrolase activity screening on different extreme halophilic isolates from Argentina salterns

Isolate	Related strain	Protease		Amylase	Cellulase	Lipase	DNAse
		Cas	Hem				
C	*Haloarcula argentinensis*	+	+	+	−	+	−
G	*Haloarcula japonica*	+	+	+	−	+	−
V	*Haloarcula vallismortis*	+	−	+	+	+	−
F	*Halorubrum tebenquichense*	+	−	+	−	−	−
2	*Halobacterium salinarum*	+	−	−	−	−	−
E	*Halobacterium* sp.	+	−	−	−	−	−
P	*Halobacterium piscisalsi*	+	−	+	+	−	−
Kr	*Salinibacter ruber* M8	−	−	−	−	−	−
Kb	*Salicola* sp.	+	−	−	+	+	−

Microorganisms were streaked onto SW agar plate co-polymerized with 1 % of the corresponding substrate: casein (Cas, skim milk) or hemoglobin (Hem) for proteases; soluble starch for amylases; carboxymethyl cellulose (CMC) for cellulases; olive oil for lipases; salmon sperm DNA for nucleases. The plates were incubated at 37 °C for 7 days and the enzyme activity was determined as indicated in "Materials and methods"

phylogenetic analysis of the isolates, DNA was extracted from pure cultures and used as template to amplify fragments of the 16S rRNA gene by PCR. The outcome of this analysis showed that seven archaeal and two bacterial representatives had been isolated.

The isolated archaea corresponded to family *Halobacteriaceae* and were closely related to three genera (Table 1): *Haloarcula*: three isolates, C (*Haloarcula argentinensis*, Ihara et al. 1997), G (*Haloarcula japonica,* Takashina et al. 1990) and V (*Haloarcula vallismortis,* Gonzalez et al. 1978); *Halobacterium*: three isolates, 2 (*Halobacterium salinarum*), E (*Halobacterium* sp.) and P (*Halobacterium piscisalsi*, Yachai et al. 2008); and *Halorubrum*: one isolate, F (*Halorubrum tebenquichense,* Lizama et al. 2002). These organisms are within the most widely distributed archaea inhabiting saline environments (Benlloch et al. 2002; Birbir et al. 2007; Ochsenreiter et al. 2002; Sabet et al. 2009). From *Bacteria* domain, Kr (*Salinibacter ruber* (Bacteroidetes), Antón et al. 2002), and Kb (*Salicola* sp., Gamma proteobacteria) were isolated. These microorganisms, together with *Halomonas* strains, are typical bacterial representatives in these environments (Antón et al. 2002; Borsodi et al. 2013; Maturrano et al. 2006; Mutlu et al. 2008; Sabet et al. 2009). Indeed, members belonging to both genera *Salinibacter* and *Salicola* are known to dominate in bacterial communities from hypersaline water bodies reaching salt concentrations close to halite saturation.

Hydrolytic enzymes

To measure the extracellular hydrolytic activities of the isolated halophiles, the microorganisms were grown onto agar plates supplemented with the appropriate substrate and incubated until hydrolysis was evident (halos). The results are shown in Table 2 and Fig. S1.

Proteases (E.C. 3.4) hydrolyze peptide bonds releasing peptides and/or amino acids. These enzymes have been the most extensively studied as they are applied in a wide variety of industrial processes. The fact that halophilic proteases are active/stable in solutions containing

high salt concentrations (> 2 M NaCl), at a wide pH range (5–10), moderate to high temperatures (37–80 °C) and in presence of organic solvents, makes them suitable candidates for biotechnological and industrial processes (De Castro et al. 2006; Delgado-García et al. 2012; Litchfield 2011; Moreno et al. 2013; Oren 2010; Ruiz et al. 2010). The halophiles isolated in this work have been previously described as capable of gelatin digestion with the exception of *Hrr. tebenchiquense*, *Har. japonica and Har. vallismortis* (Antón et al. 2002; Gonzalez et al. 1978; Lizama et al. 2002, Maturrano et al. 2006; Takashima et al. 1990, Yachai et al. 2008). *Har. argentinensis* had not been tested before (Ihara et al. 1997). The results in Table 2 and Fig. S1 show that, with the exception of isolate Kr (*S. ruber)*, all the strains exhibited extracellular protease activity in presence of casein (skim milk). In addition, isolates C and G (*Har. argentinensis* and *Har. japonica,* respectively) hydrolyzed hemoglobin. Considering that the haloproteases reported so far are of the serine-protease type (Enache and Kamekura 2010), the positive response of isolates F, G, V and C (*Hrr. tebenquichense*, *Har. japonica, Har. vallismortis* and *Har. argentinensis,* respectively) made these Argentinean strains good candidates for further studies to search for novel proteases.

Amylases (E.C.3.2.1.1) and cellulases (E.C.3.2.1.4) break down the complex structures of starch and cellulose, respectively. While a wide variety of halophilic amylase producers have been described (Enache and Kamekura 2010; Litchfield 2011; Ventosa et al. 2005), a few cellulolytic halophiles have been reported so far (Cojoc et al. 2009; Li and Yu 2013; Venkatachalam et al. 2014). Table 2 shows that three of the tested strains, Kb, V and P (*Salicola* sp., *Har. vallismortis* and *Hbt. piscisalsi)* degraded CMC when it was co-polymerized in the medium whereas isolates C, G, V, P, F showed the capacity to degrade starch. Li and Yu (2013) reported that the cellulase from *Haloarcula* sp. strain LLSG7 is used in bioethanol production, due to its optimal conditions of pH, salt and temperature, solvent stability and substrate specificity.

The presence of amylolytic and cellulolytic activities in isolates V and P increases their potential utility in applications that require the combination of both enzymatic activities as, for example, the bioconversion of renewable high molecular polysaccharides present in algae (Brányiková et al. 2011; Peat and Turvey 1965) to sugars, especially for the production of ethanol by a fermentation process in large-scale industries (Cherry and Fidantsef 2003; Nguyen and Vu 2012).

Lipases (triacylglycerol hydrolases, E.C.3.1.1.3) catalyze the hydrolysis of long-chain acylglycerols in aqueous emulsions. As they are applied in different industries (food, paper, pharmaceutical and/or cosmetic), bioremediation or as detergent additives (Hasan et al. 2006), extensive screenings for halophilic lipases have been carried out (Bhatnagar et al. 2005; Fuciños et al. 2012; Ozcan et al. 2009; Litchfield 2011; Moreno et al. 2013). To assay for the presence of this activity, the isolated strains were grown on SW agar medium supplemented with 1 % olive oil as source of triacylglycerols (triolein). Only four of the analyzed strains (C, G, V and Kb) hydrolyzed this substrate (Table 2).

Polyoxyethylene sorbitans (Tweens) have been used as substrate to measure lipase/esterase activity in different microorganisms (Flores et al. 2010; González et al. 1978; Plou et al. 1998; Tirunarayanan and Lundbeck 1968). As these compounds are esters of fatty acids, they are useful to assess a difference in substrate recognition and hydrolysis. Tween 20 and 80 are esters of lauric and oleic acids, respectively, which allow testing the enzyme preference for saturated or unsaturated fatty acids as substrate. Hydrolysis is evidenced by the presence of a precipitation halo of calcium salt around the colonies, due to the release of fatty acids. As shown in Fig. 1, isolates C, G, P, F and Kb developed precipitation halos, when they were grown onto agar plates containing Tween 20 or Tween 80, indicating the hydrolysis of both substrates. On the other hand, the halo observed around strain V only in presence of Tween 80 evidenced its capacity to degrade unsaturated fatty acids (Fig. 1). Similar results of preferential degradation were obtained when the culture medium SW was prepared with casamino acids instead of YE, with the exception of a week and variable response to Tween 20 for this archaeon (data not shown). These results suggest the presence of at least two different activities, one unspecific lipase/esterase activity evidenced by Tween 20 and Tween 80 degradation by two *Haloarcula* related isolates (C and G), F and Kb, and another specific for unsaturated fatty acids in isolate V. The selectivity of isolate V makes it an interesting subject for future research due its potential application in food industries that require hydrolysis/esterification of unsaturated fatty acids such as processing and flavor improvement (Hasan et al. 2006). Extracellular esterase and lipase activities have also been reported for the related species *Har. marismortui*, although the enzymes have not been purified yet (Camacho et al. 2009).

Considering the results obtained with Tween and olive oil (Fig. 1, Table 2) it can be speculated that the activities detected in isolates C, G, V and Kb may be attributed to either two different enzymes (lipase and esterase) or to an extracellular lipase exhibiting both activities. However, it is likely that the activity detected in isolates P and F may correspond to an extracellular esterase since these microorganisms failed to degrade olive oil. An intracellular esterase activity was characterized for *Salicola* C10 by Moreno and coworkers (Moreno et al. 2009); however, to the best of our knowledge, there is no information on the occurrence of extracellular lipases in this microorganism.

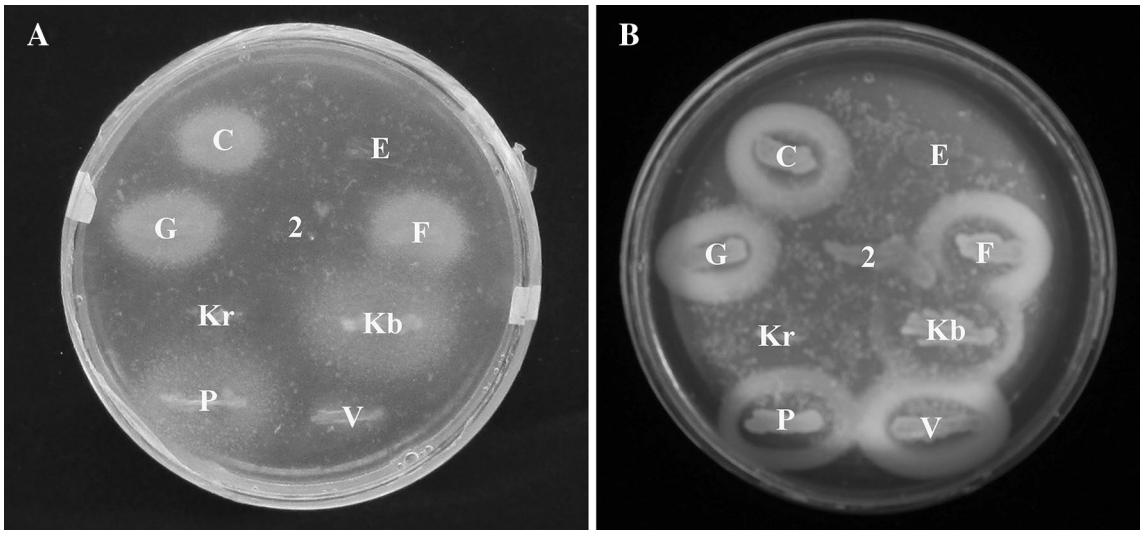

Fig. 1 Substrate specificity of lipase/esterase activity using Tween 20 and 80. Microorganisms were streaked in SW agar plate co-polymerized with 1 % (w/v) of the substrate. **a** Tween 20: lauric acid esters. **b** Tween 80: oleic acid esters. The plates were incubated a 37 °C for 7 days and the activity was determined by the presence of precipitation halos surrounding the streak. The isolates are affiliated as follows: *C*, *Har. argentinensis; E*, *Halobacterium* sp.; *G*, *Har. japonica*; *2*, *Hbt. salinarum*; *F*, *Hrr. tebenquichense*; *Kr*, *S. ruber*; *Kb*, *Salicola* sp.; *P*, *Hbt. piscisalsi*; *V*, *Har. vallismortis*

Although, halophilic microorganisms are known to produce extracellular nuclease activity (Delgado-Garcia et al. 2012; Oren 2010) none of the strains tested was able to degrade DNA, at least under the conditions used in this study (Table 2).

Bioactive compounds

Surfactants

Biosurfactants molecules influence microbial physiological behavior by their property to decrease surface and interfacial tensions between different phases. When purified they are useful in different industries (Reis et al. 2013; van Hamme et al. 2006; Singh et al. 2007) due to their low toxicity, high biodegradability, selectivity and specific activity, in addition to the low cost production. The screening for biosurfactant producers has increased in the last decade; however, halophilic microorganisms have been relatively poorly investigated (Cameotra and Makkar 1998; Litchfield 2011; Margesin and Schinner 2001; Satpute et al. 2010). Recent studies showed that halophilic biosurfactants remain stable in a broad pH range (5–12) and high salt concentration (3 M), whereas screenings in hypersaline environments (soda lakes, oil fields and solar ponds) are increasing the number of surfactant producers (Djeridi et al. 2013; Kebbouche-Gana et al. 2009, 2013; Sarafin et al. 2014; Selim et al. 2012; Wu et al. 2014). Several reports show the application of surfactants produced by members of *Halomonas* genus in industry and biomedicine (Calvo et al. 2002; Donio et al. 2013; Llamas et al. 2012) making worth efforts to screen for novel biosurfactant producers in high salinity habitats.

To examine the occurrence of biosurfactants, emulsifying assays were carried out with cell-free culture media of the isolates. Among the different genera to which the isolates were affiliated, only one *Haloarcula* strain was described as surfactant producer (Kebbouche-Gana et al. 2009).

The ability to produce emulsions was tested with two different substrates, olive oil and xylenes, while the commercial surfactants SDS and Triton X-100 were used as positive controls (Fig. 2). The results show that all tested strains, except isolate E (*Halobacterium* sp.), emulsified olive oil. While all the surfactant producers were more active than the anionic surfactant used as control (1 % w/v SDS), isolates P, F, G, V and Kb (related to *Hbt. piscisalsi, Hrr. tebenquichense, Har. japonica, Har. vallismortis* and *Salicola* sp., respectively) were also more effective than the no ionic surfactant (1 % v/v Triton X-100) (Fig. 2a). On the other hand, when xylene was used as substrate, cell-free medium from strains Kb, E, G, V, P and F exhibited higher emulsifying capacity than SDS while Triton X-100 did not have any effect (Fig. 2c).

The stability of the emulsions generated with both substrates was followed at room temperature for 15 days maintaining stable for this time period and up to at least 45 days (data not shown) (Fig. 2b, d). In presence of olive oil the cell-free media from all the strains behaved similarly showing a decrease in the range of 15–30 % after the first 24 h

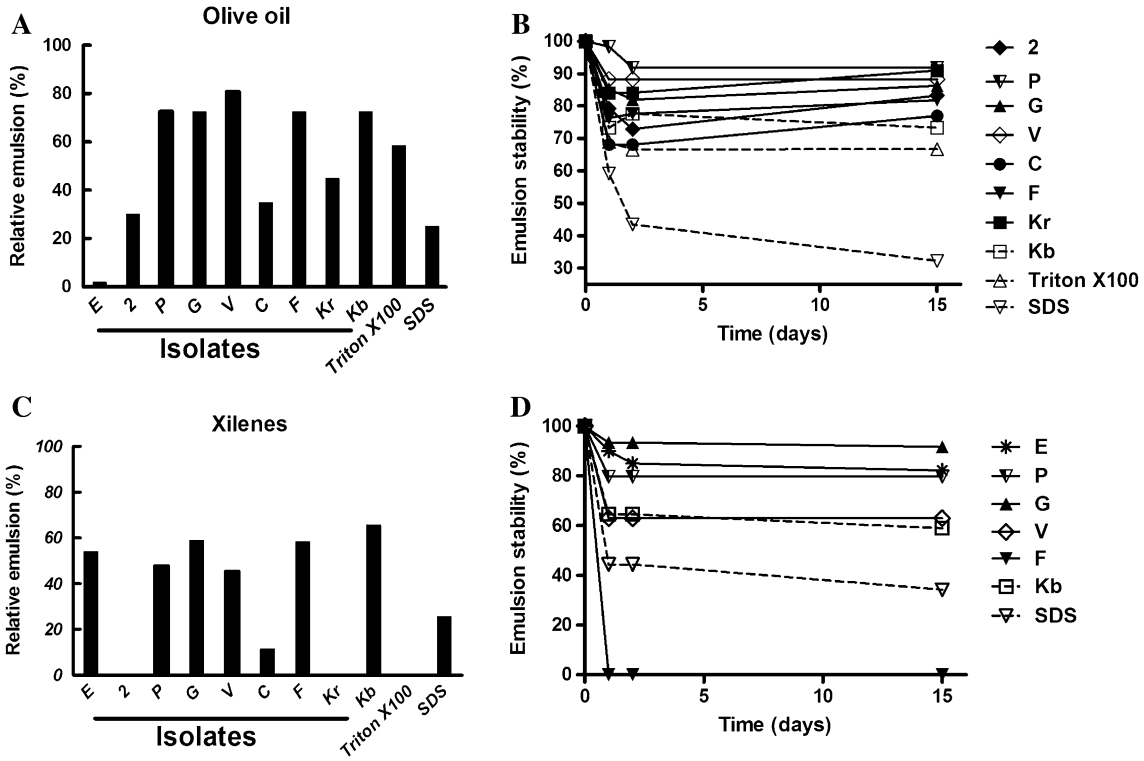

Fig. 2 Biosurfactant production by halophilic isolates and emulsion stability over time. The strains were incubated in SW until reach $DO_{600} = 1$ and cell-free media was used as biosurfactant source. Olive oil (**a**, **b**) or xilenes (**c**, **d**) were used as substrate in the assay. Equal volumes of cell-free medium and substrate were vortexed at high speed for 2 min and kept for 2 h prior measurement (**a**, **c**). Then, the emulsions were kept at RT and the stability was followed for 15 days (**b**, **d**). The emulsification activity is defined as the height of the emulsion layer divided by total height and expressed as percentage

and remaining stable for up to 45 days. It is noteworthy that the olive oil emulsions produced with the surfactants synthesized by the halophilic isolates were more stable than those produced by SDS, which is widely used in industries. Under the conditions tested in this study (medium containing high salt concentration), SDS showed a decrease of 40–50 % of its emulsifying activity after 48 h.

When xylene was used as substrate, the emulsion produced by the cell-free medium derived from cultures of E, P and G decreased around 15 %, whereas a major decrease was observed with Kb, V and SDS (40 and 60 %, respectively) for the first 24 h and then remained stable. It was curious that the emulsion produced by strain F was completely lost after 24 h.

These results show that the biosurfactant produced by isolates G, V, P and Kb could emulsify both aromatic compounds and long-chain hydrocarbons, whereas those synthesized by strains Kr, C, 2 and F or E specifically emulsified long-chain hydrocarbons or aromatic compounds, respectively, better than commercial products such as SDS and Triton X-100.

To determine if the emulsifying activity of isolate G onto both substrates was produced by one or more compounds, we attempted to isolate these molecules using acidic precipitation of the cell-free culture media. Stable foam-olive oil was obtained only by the suspended pellet, whereas xylene was emulsified by both the acidic and the neutralized supernatant. These results suggest the presence of at least two different active molecules in this haloarchaeon.

In view of the wide range of industrial applications of biosurfactants and the demanding low cost for high yield production (Reis et al. 2013; Singh et al. 2007), different growth conditions were analyzed with the aim of improving surfactant production. Microorganisms were cultivated under two temperatures (30 and 55 °C) or salinities (2.5 and 5.0 M NaCl) observing an increase of emulsifying activity only with strain 2 at 55 °C, whereas a similar response to those obtained at normal condition (37 °C, 3.5 M NaCl) was observed with the rest of the strains. When isolate C was growth in SW media with casamino acids a twofold increase in emulsifying activity was observed in presence of olive oil.

Between the multiple applications of biosurfactants in different industries, those related to environmental remediation are the most attracting. For example, the brackish water (salty water with high content of aliphatic

Table 3 Halocin production and sensitivity of the halophilic isolates from two Argentinean salterns

Producer	Lawn								
	C	G	V	F	2	E	P	Kr	Kb
C		–	+	+	+	±	+	–	–
G	–		+	+	+	–	–	–	–
V	–	–		–	–	–	–	–	–
F	–	–	–		–	–	–	–	–
2	–	–	–	+		–	+	–	–
E	–	–	–	+	+		–	–	–
P	–	–	–	+	+	–		–	–
Kr	–	–	–	–	–	–	–		–
Kb	–	–	–	–	–	–	–	–	

The indicator strains were mixed with top agar (0.7 %) and once solidified, drops of the test microorganisms were spotted over. The plates were incubated until a homogenous microbial lawn was observed. Halocin production was considered positive (+) when clear inhibition zones appeared around the spotted strain. The isolates are affiliated as follows C: *Har. argentinensis*, E: *Halobacterium*sp., G: *Har. japonica,* 2: *Hbt. salinarum,* F: *Hrr. tebenquichense,* Kr: *S. ruber,* Kb: *Salicola* sp, P: *Hbt. piscisalsi,* V: *Har. vallismortis*

compounds and aromatics as benzene, ethylbenzene, toluene and xylenes) generated during oil and gas extraction on oilfields are difficult to be remediated due to the low solubility of hydrocarbon and the high salt content. The availability of compounds able to differentially increase their solubility as those produced by strains G or F and the progress in the knowledge of degradation pathways present in halophilic microorganisms during the last decade could help to design better strategies for bioremediation in hypersaline environments (Fathepure 2014).

Halocins

Halocins are small proteins or peptides produced by halophilic microorganisms that are secreted to the extracellular medium and exhibit antimicrobial activity (O'Connor and Shand 2002). These molecules are divided into microhalocins and halocins, depending on their molecular mass (3–5 to 35 kDa). They inhibit growth in different ways, but the molecular mechanisms of these compounds are poorly understood. The effectiveness of halocins is variable as they can target a broad spectrum of genera as well as only closely related species (Atanasova et al. 2013; Imadalou-Idres et al. 2013; O'Connor and Shand 2002). In addition, inter-domains interactions have been established showing the growth inhibition of *Halorubrum* sp. by *Salicola* sp. meanwhile several bacteria were inhibited by *Haloferax* sp. (Atanasova et al. 2013). Despite it was proposed that halocin production is a feature shared by all halophilic microorganisms, at present only a few number of these molecules have been characterized (Kavitha et al. 2011; Meseguer and Rodríguez Valera 1985; O'Connor and Shand 2002; Price and Shand 2000; Torreblanca et al. 1994).

Besides their importance in the control of natural microbial population in saline environments, the potential application of antimicrobial compounds such as halocins in industrial activities is in the focus as they could replace the antibiotics that are generating resistance in infectious microorganisms. Bioactive molecules with antimicrobial activity isolated from extremophiles may offer the possibility of discovering novel mechanisms against undesirable microorganisms and could be applied in both food and health industries. For instance, halocin H6 from *Haloferax gibbonsii* which acts as a Na^+/H^+ antiporter inhibitor was reported as an effective cardio-protector on the ischemic and reperfused myocardium in dogs (Lequerica et al. 2006).

In this study, we tested halophilic isolates for the production of antimicrobial molecules. For this, each microorganism was tested against each other and the growth inhibition was analyzed by the presence of typical inhibition halos onto solid medium. As shown in Table 3 and Fig. S2, several strains exhibited halocin activity against the other isolates. Both isolates corresponding to *Bacteria* domain were resistant to all archaeal isolates and the latter were also resistant to bacterial strains, indicating that no inter-domain interaction existed. Surprisingly, the strains related to *Haloarcula* genus, C and G, were the most effective producers, inhibiting growth of almost all the archaeal isolates, including strain V. Also, they produced the biggest inhibition zones onto the indicators lawns and were resistant to all the other strains (Fig. S2). Furthermore, when they were used as indicator lawn none of the other isolates had grown, with the exception of themselves and the bacterial strains, confirming the potential of the antagonistic activity.

This fact resulted notorious because at present there is no information about *Haloarcula* spp. halocin-producers,

except the report by Atanasova et al. (2013) in which one strain of *Haloarcula* genus inhibited growth of different related species. However, in contrast to this study, in which interactions were observed between microorganisms from the same saltern, the intra-genus inhibition reported was produced between isolates from different saline environments (Atanasova et al. 2013).

Among the Argentinean isolates, other halocin-producers belonged to *Halobacterium* genus: P, 2 and E (Table 3). They were effective against isolate F, which resulted to be the most sensitive strain. But the archaeon 2 was also sensitive to E and P strains, suggesting a competition among related species.

These results confirm the presence of bioactive compounds with antimicrobial activity with different targets in the microorganisms inhabiting these salterns, re-enforcing the idea of a dynamic population where cell density may be controlled to some extent by these molecules.

Screening the halocin production in hypersaline environments around the world carried out in the lasts years described members belonging to genera *Natrinema* and *Halorubrum* and the archaeon *Haloferax volcanii* as producers of halocins with activity against a broad number of microorganisms (Atanasova et al. 2013; Imadalou-Idres et al. 2013; Kavitha et al. 2011). Thus, the results from Argentinean salterns contribute to the pool of producers, incorporating the microorganisms mentioned above.

Conclusions

The analysis performed in this study shows the multiple potential of the halophilic microorganisms inhabiting Salitral Negro and Colorada Grande Argentinean salterns, as a broad spectrum of enzymatic activities and metabolites of biotechnological application. All the halophiles tested were able to produce several, if not all the activities assayed, thus reinforcing the idea of screening extreme environments as a source of potentially useful and novel molecules applicable in biotechnology and industry.

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