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ORIGINAL PAPER

Resistance of a *Halobacterium salinarum* isolate from a solar saltern to cadmium, lead, nickel, zinc, and copper

Houda Baati · Mariem Siala · Chafai Azri · Emna Ammar · Christopher Dunlap · Mohamed Trigui

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Abstract The current study focuses on the tolerance of a strain of *Halobacterium salinarum* isolated from Sfax solar saltern (Tunisia) towards cadmium (Cd), lead (Pb), nickel (Ni), zinc (Zn), and copper (Cu) by using agar dilution methods in complex and minimal media. The results showed the least inhibitory metals based on Minimum Inhibitory Concentrations (MICs) were lead (MIC = 4.5 mM), cadmium (MIC = 4 mM), and nickel (MIC = 2.5 mM) in complex medium. The MICs of these metals were more inhibitory (MIC < 2 mM) in the other tested media. The archaeal strain revealed a high sensitivity for copper and zinc, with MICs below 0.5 mM for both metals. Growth kinetics in complex and minimal media showed the strain to be more sensitive to the metals in liquid media than in solid media. The growth kinetic assays indicated the presence of selected heavy metals resulted in a lower growth rate and lower total cell mass relative to the control. Despite that cadmium and lead are nonessential and have no nutrient value, they were the most tolerated metals by *H. salinarum* strain. In addition, pigment intensity in the strain was inhibited by the presence of the heavy metals relative to the control.

Keywords Solar saltern · *Halobacterium salinarum* · Heavy metals · Minimal inhibitory concentrations · Tolerance · Growth kinetics · Generation time

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Introduction

Heavy metal contamination is commonly found in saline and hypersaline soil environments due to the same processes that causes salinity; accumulation through evaporation or as a result of industrial activities (Voica et al. 2016). Natural habitats, such as estuaries, can also be contaminated with heavy metals and salts, through similar processes allowing these compounds to accumulate. These problems are exacerbated by anthropogenic activities, such as urbanization or industrialization, leading to greater

introduction of these compounds into ecosystems (Srivastava and Kowshik 2013). The combination of high salt and heavy metal stress influence the microbiota found in these locations. Microorganisms must be able to tolerate these stressors (Rios et al. 1998). Bioremediation of hypersaline environments with conventional microorganisms is not possible due to the high salinities of these environments. High saline levels may disrupt microorganisms osmotic balance and denature metabolic enzymes (Erdogmus et al. 2013). Therefore, there is a pressing need to isolate extremely halophilic microorganisms for bioremediation of polluted hypersaline environments. Research on the tolerance of heavy metal contamination on microorganisms is mostly limited to *Bacteria*, while the closely related *Archaea* microorganisms are much less studied. Therefore, halophilic *Archaea* represent an unexplored option of high salt and heavy metal tolerant microbes. In addition, halophilic *Archaea* have been shown to tolerate high temperatures, which makes them polyextremophiles. These characteristics are highly desired when searching for microorganisms to handle the conditions in extremely stressful environments (Kottemann et al. 2005; Oren 2012; Bonete et al. 2015). Currently, many locations are becoming contaminated and require bioremediations. Extremophilic *Archaea* have the potential to serve this role and have already been selected as agents for bioremediation of industrial wastewaters contaminated with salt, heavy metals and other abiotic stresses (Zhuang et al. 2010; Krzmarzick et al. 2018). Halophilic *Archaea* have developed a variety of resistance mechanisms to survive environments contaminated with heavy metals (Wang et al. 2004; Kaur et al. 2006; Bini 2010; Bonete et al. 2015). Haloarchaea synthesize exopolysaccharides (EPS) to protect themselves under adverse conditions such as nutrient starvation, temperature fluctuation, and the presence of heavy metals (Poli et al. 2011; Völkel et al. 2020). They may prove to be an important tool in bioremediation of heavy metal contamination in extreme saline ecosystems (Oren 2012; Voica et al. 2016). The goal of this research was to isolate and identify archaeal microorganisms tolerant to heavy metals and build on the development of these organisms to treat and bioremediate sites (Nieto et al. 1987; Unz and Shuttleworth 1996; Rios et al. 1998; Enache et al. 2000; Gabballa et al. 2003; Amoozegar et al. 2005; Al-Momani et al. 2007; Popescu and Dumitru 2009; Bini 2010; Chaudhary et al. 2014; Das et al. 2014; Salgaonkar et al. 2016; Voica et al. 2016; Völkel et al. 2018).

The solar saltern of Sfax (Tunisia), is a multi-pond facility located near the industrial region of the city. This has led the facility to become contaminated with particles from industrial particulate fallouts highly enriched with heavy metals (cadmium, lead, nickel, zinc, and copper; Azri et al. 2007, 2010). The area is home to factories involved in phosphate treatment (SIAPE plant), soap manufacturing (SIOS-ZITEX), and lead smelting (FP Sfax Sud). These factories are collocated near the saltern, on the southern edge of the city and are harmful to the environment because they emit pollutants regularly exceeding Tunisian standards on sulfur oxides (SOx) and particulate matter (JICA 1993; Azri et al. 2009). Additionally, the solar saltern ponds are exposed to wind borne pollutants from both marine and land sources due to the wind patterns in the region (Bahloul et al. 2015a). Westerly winds dominate the area and regularly expose the region's factory plumes (Bahloul et al. 2015b; Dammak et al. 2016). Several studies have explored the physical, chemical, biological, and microbial biodiversity of the Sfax saltern (Baati et al. 2008, 2012; Trigui et al. 2011; Boujelben et al. 2014), but none have explored the heavy metal tolerance of *Archaea*. Furthermore, to this date, there are no reports on heavy metals tolerance of extremely halophilic *Archaea* isolated from solar salterns. Therefore, this study is aimed at evaluating the resistance of an archaeal strain affiliated with *H. salinarum* against various concentrations of selected heavy metals (cadmium, lead, nickel, zinc, and copper).

Materials and methods

Site description

The Sfax solar saltern sampled during this study is located in the southern edge of the city (Central-Eastern coast of Tunisia, about 34°39 North–10°42 East). The salinities of the salterns vary from seawater to saturated salt solutions. The evaporation ponds are shallow (20–70 cm deep), connected by a series of canals and pipelines. They were designed to produce NaCl crystals through the evaporation of seawater. During the evaporation process, the salt brines become

progressively more concentrated until they reach saturation and the salt crystallizes.

Strain selection and cultivation conditions

The selected archaeal strain for this study was isolated from sediment samples collected aseptically during May 2017 from the Sfax solar saltern (Tunisia) as described previously (Baati et al. 2020). The archaeal strain was selected among many isolates due to its higher resistance to tested heavy metals. It grew at 37 °C in DSC-97 medium containing (g L^{-1}): yeast extract, 10; casamino acids, 7.5; NaCl, 250; $MgSO_4$·$7H_2O$, 20; KCl, 2; and trisodium citrate, 3 (DasSarma et al. 1995). The pH was adjusted to 6. The strain was stored at − 80 °C in 20% glycerol (w v^{-1}).

Molecular identification of the selected archaeal strain

Genomic DNA extraction

The cells were harvested by centrifugation (8000 g for 30 min), and the genomic DNA was extracted using Genomic DNA Purification Kit (NucleoSpin Tissue Kit, macherey–Nagel) according to the manufacturer's protocol. The kit protocol was followed by an ethanol precipitation to further clean the DNA.

PCR amplification and DNA sequencing

PCR amplification of the 16S rRNA gene was performed using TaKaRa Ex TaqTM (2.5 units, Promega) in 50 µL reaction buffer, containing 2 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 20 µL of each primer, and 5 µL of 10× Ex Taq bufferTM. The primers used were archaeal-specific primer 21 F (DeLong 1992) combined with the universal reverse primer 1390R (Zheng et al. 1996). The PCR amplification was carried out according to the following program: initial denaturation at 94 °C for 5 min and 24 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 59 °C for 1 min, and extension at 72 °C for 1.5 min. A final elongation step was performed for 15 min. The PCR product was sequenced using an automated Sanger sequencer at the DNA core facility at the National Center for Agricultural Utilization Research center in Peoria, IL, USA.

Phylogenetic analysis of 16S rRNA

The resulting 16S rRNA gene sequence obtained was compared to those available at the EzBiocloud (Yoon et al. 2017). The retrieved data were aligned, and nucleotide substitution model testing was performed using MEGA-X software (Kumar et al. 2018). The neighbor-joining tree was determined using the Tamura-Nei model (0.40, gamma distributed with invariant sites) based on model testing under MEGA X (Kumar et al. 2018). Measures of bootstrap support for internal branches were obtained from 1500 pseudoreplicates. The sequence data obtained in this study has been submitted to EMBL/GenBank databases under accession number MT332425.

Determination of Minimum Inhibitory Concentrations (MICs)

The MICs of selected heavy metals (cadmium, lead, nickel, zinc and copper) were determined by gradually increasing the concentration of each metal in the media until the culture ceased to grow. Growth and heavy metals tolerance of the selected archaeal strain were studied in three different media. The first medium was DSC-97 medium. The second medium was the complex nutrient rich medium (NTYE, NaCl Tryptone Yeast Extract) containing (g L^{-1}) NaCl, 250; $MgSO_4$·$7H_2O$, 20; yeast extract, 3; tryptone, 5; KCl, 5; and agar 20 at pH 6 (Braganca and Furtado 2009). The third medium was the synthetic mineral medium referred to as NGSM comprising of (g L^{-1}) of NaCl, 200; $MgCl_2$·$6H_2O$, 13; $CaCl_2$·$6H_2O$, 1; KCl, 4; $NaHCO_3$, 0.2; NH_4Cl, 2; $FeCl_3$·$6H_2O$, 0.005; KH_2PO_4, 0.5; yeast extract, 1; glucose, 2; and agar 20 at pH 6 (Salgaonkar et al. 2012). The main difference between DSC-97 and NTYE is the presence of trisodium citrate in DSC-97 medium, which can support the growth of fastidious organisms while NTYE does not.

Each type of the media was prepared with different metals ($ZnSO_4$·$6H_2O$; $NiSO_4$·$6H_2O$; $CuSO_4$·$5H_2O$; $PbCl_2$·$2H_20$; and $CdCl_2$·$2H_2O$) at the following concentrations (0.5-, 1-, 2-, and 4-mM for $ZnSO_4$·$6H_2O$; 0.5-, 1-, 2-, 2.5-, and 4-mM for $NiSO_4$·$6H_2O$; 0.1-, 0.2-, 0.4-, and 0.5-mM for $CuSO_4$·$5H_2O$; and 0.5-, 1-, 2-, 4-, 4.5-, and 5-mM for $PbCl_2$·$2H_2O$). All of the stock solutions prepared in deionized water were filter sterilized with 0.22 µm membrane filters (Millipore).

Solid media containing the different concentrations of heavy metals were inoculated with 100 µL of haloarchaeal cultures obtained in the exponential growth phase. The plates were incubated at 37 °C for 15 days. Media without heavy metals were inoculated with the haloarchaeal strains and used as controls. The minimum metal concentrations that inhibited growth of the *Archaea* was reported as MICs (Minimum Inhibitory concentrations).

Growth kinetics of the selected archaeal strain in the presence of heavy metals

The strain tolerance to heavy metals was also tested and analysed in liquid media. Three media, DSC-97, complex nutrient rich medium (NTYE), and the minimal synthetic mineral medium (NGSM) were used. $ZnSO_4{\cdot}6H_2O$; $NiSO_4{\cdot}6H_2O$; $CuSO_4{\cdot}5H_2O$; $PbCl_2{\cdot}2H_20$; and $CdCl_2{\cdot}2H_2O$ at several concentrations (0.5-, 1-, 2-, 4-mM for cadmium and lead; 0.5-, 1-, 2-mM for nickel and zinc and 0.1-, 0.2-, 0.4-mM for copper) were used for DSC-97, NTYE and NGSM. 1% of the mid log culture was inoculated in 100 ml of each media. All of the flasks were incubated at 37 °C and at 110 centrifugal force (*g*) into a shaking incubator (Daihan Lab Tech CO, LTD) for a period of 10 days. A control flask without heavy metal was also prepared for each media. The culture growth was monitored at 24 h intervals at 600 nm by UV–Vis spectrophotometer (Genesys 10 SUV–Vis). The experiment was repeated three times to ensure reproducibility. The growth rate was calculated, using two measures during the exponential phase, according to the formula (1) (Berney et al. 2006):

$$\mu max = \Delta Ln/\Delta t = (LnOD_2 - LnOD_1)/t_2 - t_1 \quad (1)$$

where OD_2 and OD_1 are the OD_{600} at times t_1 and t_2 (day), respectively.

The generation time (t) was calculated with the formula (2):

$$t = Ln2/\mu max \quad (2)$$

Growth at different NaCl concentrations and pH

Growth was studied at various NaCl concentrations ranging from 200 to 350 g L^{-1} and at pH range 5–9 in presence of 1 mM of cadmium in DCS-97, NTYE, and NGSM media. Controls without heavy metals were maintained under the same conditions. The experiment was repeated thrice to ensure reproducibility.

Pigments extraction and analysis

Pigments were extracted, after harvesting the cells, by centrifugation at 8000×*g* for 20 min at 4 °C with 10 mL of pure acetone (5% BHT). The mixture was vortexed until the entire pigment (orange-red at 8000×*g* for 20 min) was extracted in the solvent. The solvent fraction containing the pigments was separated from the cell debris by centrifugation at 8000×*g* for 10 min. The supernatant was then scanned between 350 and 650 nm using a UV–Vis spectrophotometer (Genesys 10 SUV–Vis).

Statistical analysis

The mean values and standard deviation (± SD) of three replicates were calculated using Microsoft Excel 2016. The analysis of variance (two-way ANOVA) was performed (using also Microsoft Excel 2016) in order to assess the impact of two independent factors (1st independent factors: pH and NaCl and 2nd independent one: media "DSC-97, NTYE and NGSM") on the archaeal strain growth (dependent variable) in presence of 1 mM of cadmium. ANOVA was followed by the Lowest Significant Differences (LSD).

Results and discussion

Previous works related to heavy metals tolerance of prokaryotic flora (*Bacteria* and *Archaea*) in the shallow sediments of Sfax solar saltern ponds showed the strains of *Archaea* isolated from the ponds with the greatest contamination were able to tolerant high concentrations of lead, cadmium, and nickel from 2.5- to 4.5-mM. For copper and zinc, the *Archaea* resistance levels did not exceed 1 mM (Baati et al. 2020). The heavy metal resistant strain from the collection was identified as *H. salinarum* by 16S rRNA gene sequencing (Fig. 1). This study highlights the growth kinetics and pigmentation scans in complex (DSC-97 and NTYE) and minimal (NGSM) media of archaeal strains against different metal concentrations.

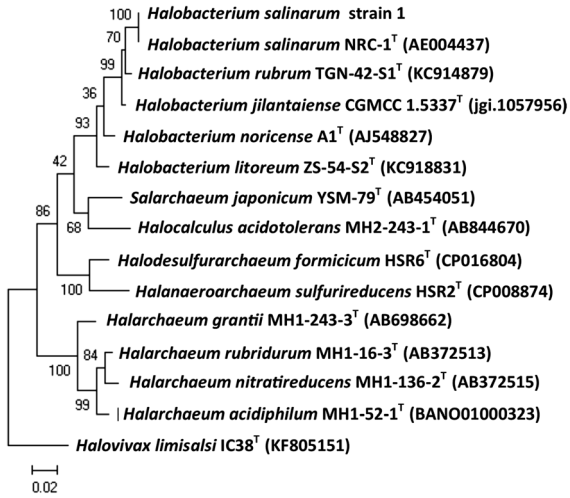

Fig. 1 Phylogenetic tree based on 16S rRNA sequences of the selected halophilic archaeal strain and other related archaeal sequences previously published in the databases was determined by the neighbor-joining method using the Tamura-Nei model (0.40, gamma distributed with invariant sites) based on model testing under MEGA X. *Halovivax limisalsi* IC38^T (KF805151) is used as an outgroup

Heavy metal MICs for *H. salinarum* in complex and minimal media

Based on Minimal Inhibitory Concentrations (MICs) in three different solid media (DSC-97, NTYE, and NGSM), *H. salinarum* was characterised for its tolerance pattern to cadmium, lead, nickel, zinc, and copper. The results presented in Table 1 showed that higher MIC values were attributed to lead (MIC = 4.5 mM), cadmium (MIC = 4 mM), and nickel (MIC = 2.5 mM) in DSC-97 medium, but in NTYE and NGSM media the MIC values decreased until 2 mM for those heavy metals. The highest toxicities of metals were found with copper and zinc because of their lower MIC values never exceeding 0.5 mM. Nieto et al. (1987) demonstrated that the presence of high concentrations of NaCl increases the toxicity of Zn due to the formation of $ZnCl^-$ species which is more toxic than the cationic Zn^{2+}. Additionally, the haloarchaeal MICs of cadmium, zinc, nickel, and copper were 0.05–2.5 mM, 0.05–0.5 mM, 0.1–2.5 mM, and 1–2.5 mM, respectively. For lead, the MIC ranged between 5 and 20 mM. Williams et al. (2013) reported that *Halobacterium saccharovorum* can tolerate only up to 0.001 of cadmium and 0.01 mM of zinc. Das et al. (2014) showed the best

Table 1 Minimum inhibitory concentration (MIC) of the different heavy metals on *Halobacterium salinarum* grown in DSC-97, complex (NTYE), and minimal (NGSM) media

Growth media	DSC-97	NTYE	NGSM
Cd			
Control	+++	+++	+++
0.5 mM	+++	+++	+++
1 mM	+++	+++	+++
2 mM	++	++	+
4 mM	++	−	−
4.5 mM	−	−	−
Pb			
Control	+++	+++	+++
0.5 mM	+++	+++	+++
1 mM	+++	+++	+++
2 mM	+++	+	+
4 mM	+++	−	−
4.5 mM	+++	−	−
5 mM	−	−	−
Ni			
Control	+++	+++	+++
0.5 mM	+++	+++	+++
1 mM	+++	+++	++
2 mM	+++	+	+
2.5 mM	+	−	−
4 mM	−	−	−
Zn			
Control	+++	+++	+++
0.5 mM	+++	+++	+++
1 mM	−	−	−
2 mM	−	−	−
4 mM	−	−	−
Cu			
Control	+++	+++	+++
0.1 mM	+++	+++	+++
0.2 mM	+++	+++	+++
0.4 mM	++	++	−
0.5 mM	−	−	−

+++ Very good growth, ++ good growth, + growth, − no growth

MIC of cadmium obtained for the genera *Halococcus* and *Haloferax,* isolated from solar salterns of Goa and Tamil Nadu, was 0.5 mM but was resistant up to 4 mM levels which is higher compared to the

literature. Salgaonkar et al. (2016) demonstrated the MIC of zinc for *Haloferax* strain BBK2 was 1and 2 mM in NGSM and NTYE media, respectively. However, Popescu and Dumitru 2009 showed the MIC of such element was not exceed 1 mM in the same media. Völkel et al. (2018) showed the MICs of copper, nickel, and zinc for *H. salinarum* after 72 h cultivation were 7-, 17-, and 1-mM, respectively. Lagorce et al. (2012) demonstrated that MIC values were dependent on the type of media and substrate used. Moreno et al. (2012) showed there are no accepted standards to define a universal metal resistance. In addition, these authors noted interactions between metal ions and microbial media components can make interoperating results difficult. Due to the limitation of this technique, growth kinetics were adopted as follows.

Halobacterium salinarum growth kinetics

To test the growth of *H. salinarum* in the presence cadmium, lead, nickel, zinc, and copper, the culture was quantified by measuring the optical density (OD_{600nm}) after cultivation for 10 days in (DSC-97, NTYE, and NGSM) liquid media. For each metal, three or four concentrations up to the respective MIC were added to the cultures. As a control, growth of cells was analysed in media lacking the respective metals. The growth curves showed a reduction in OD_{600nm} values for test cultures with rising cadmium, lead, nickel, zinc, and copper concentrations, highlighting the effect of these heavy metals on *H. salinarum* cultures (Figs. 2, 3 and 4). The control in DSC-97 medium reached a maximum OD_{600nm} of 2.93 in 7 days. A maximum OD_{600nm} of 0.87 was obtained in 4 days in NTYE medium. However, in NGSM medium, a maximum OD_{600nm} was around 0.32 on the 5th day of growth.

For cadmium, *H. salinarum* was able to grow at 0.5- and 1-mM in DSC-97 medium. The cultures reached OD_{600nm} of 2.4 and 1.57, respectively. In the presence of 2 mM cadmium, the growth decreased and at 4 mM growth was significantly inhibited (Fig. 2a). In NTYE medium with 0.5 mM cadmium, the maximum OD_{600nm} of 0.45 was at day 4. In the presence of 1- and 2-mM cadmium, the OD_{600nm} value decreased and at 4 mM, growth was completely inhibited (Fig. 2b). In NGSM medium, the overall growth pattern of the strain upon exposure to 0.5 mM cadmium was similar to the control. In the presence of 1 mM, the OD_{600nm} value decreased slightly. With 2 mM, it took 9 days to attain an OD_{600nm} value of 0.174, while 4 mM completely inhibited the growth (Fig. 2c).

Regarding the addition of lead, *H. salinarum* was able to grow at 0.5-, 1-, and 2-mM in DSC-97 medium. The cultures reached OD_{600nm} ranging between 2.4 and 2.6, indicating no difference compared to the control in 10 days (OD_{600nm} = 2.77). In the presence of 4 mM lead ions, the OD_{600nm} value slightly decreased (Fig. 2d). In NTYE and NGSM media, the overall growth pattern upon exposure to 0.5- and 1-mM was similar to the control. At 2 mM lead ions in NTYE medium, the OD_{600nm} value decreased and at 4 mM the growth was completely inhibited (Fig. 2e). In NGSM medium, 2- and 4-mM lead inhibited the growth completely (Fig. 2f).

For nickel, the culture reached an OD_{600nm} of 2.806 at 0.5 mM in DSC-97 medium, which is the same as the control. In the presence of 1- and 2-mM nickel ions, the OD_{600nm} value slightly decreased (Fig. 3a). In NTYE and NGSM media, the rates of growth upon exposure to 0.5 mM was similar to the control. In the 1 mM samples, the OD_{600nm} value decreased and at 2 mM, the growth was completely inhibited (Fig. 3b, c).

In zinc tolerance assays, growth containing 0.5- and 1-mM zinc in DSC-97 medium reached OD_{600nm} at 1.01 and 0.636, respectively, which were lower than the control (OD_{600nm} = 2.77). In cultures containing 2 mM zinc, growth was significantly inhibited (Fig. 3d). In NTYE and NGSM media, at 0.5 mM zinc, the OD_{600nm} value decreased. At 1- and 2-mM zinc, the growth was completely inhibited (Fig. 3e, f).

In copper tolerance assays, growth containing 0.1 mM of copper in DSC-97 medium reached an OD_{600nm} of 2.57 after 10 days, indicating no difference compared to the control, whereas the OD_{600nm} value decreased at 0.2- and 0.4-mM (Fig. 4a). In NTYE medium, the overall growth patterns of the strain upon exposure to 0.1-, 0.2-, and 0.4-mM were similar and reached an OD_{600nm} of 0.5 after 10 days (Fig. 4b). In NGSM medium, in the presence of 0.1 mM, the OD_{600nm} value decreased (Fig. 4c).

This study shows nickel is able to slightly increase *H. salinarum's* growth if the concentration of nickel is 0.5 mM and is grown in NGSM medium only (Fig. 3c). Likewise, copper can increase the strain's growth at concentrations of 0.1- and 0.2-mM in NTYE

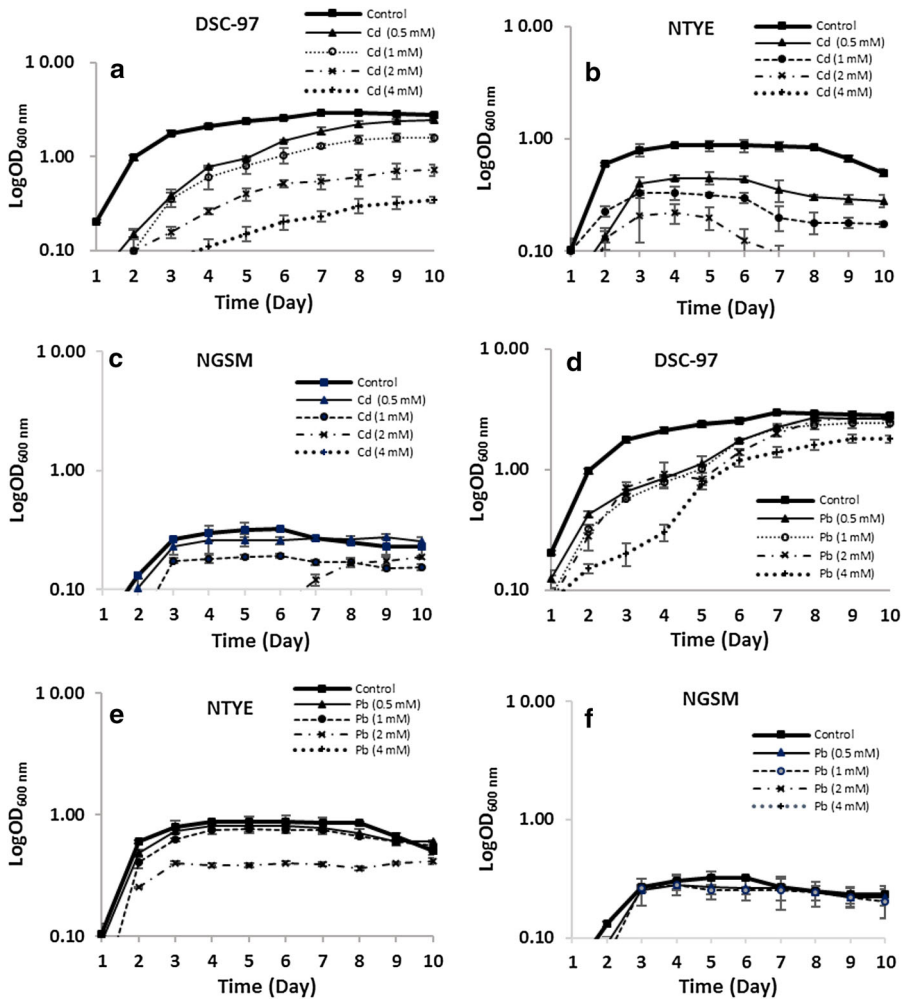

Fig. 2 Effect of cadmium and lead on growth kinetics of *H. salinarum* at different media (**a** and **d**: DSC-97; **b** and **e**: NTYE, **c** and **f**: NGSM)

after 10 days (Fig. 4b). Despite cadmium and lead being nonessential and have no nutrient value, they are the most tolerated heavy metals by *H. salinarum*. Cadmium and lead are able to increase the strain's growth at a concentration of 0.5 mM in NGSM medium after 7 days (Fig. 2c) and in NTYE medium in 10 days (Fig. 2e). Therefore, *H. salinarum* has adapted to the presence of cadmium and lead, when maximum concentrations were more than 500 and 70 times higher than previously recorded (Baati et al. 2020). Bruins et al. (2000) showed copper, nickel, and zinc are required trace elements for all living organisms and are commonly used for redox processes, serve as catalysts and structural components of various enzymes, and are important in the regulation of osmotic pressure in cells. Macomber and Hausinger (2011) and Chasapis et al. (2017) demonstrated copper and nickel ions are often used as cofactors and stabilize the active site of enzymes, such as cytochrome C oxidase (Cu) and superoxide dismutase (Ni). In addition, zinc ions are able to stabilize cell interfaces and are required cofactors for several metalloproteins (Shankar and Prasad 1998). Zinc is an essential metal for all three domains of life (*Archaea*, *Bacteria*, and eukaryote) and highly regulated in cells (Choudhury and Srivastava 2001; Andreini et al. 2006). Bini (2010) highlighted although these metals are required, when they are in excessive concentrations, they can cause damage to cells and be detrimental to life. Cadmium and lead on

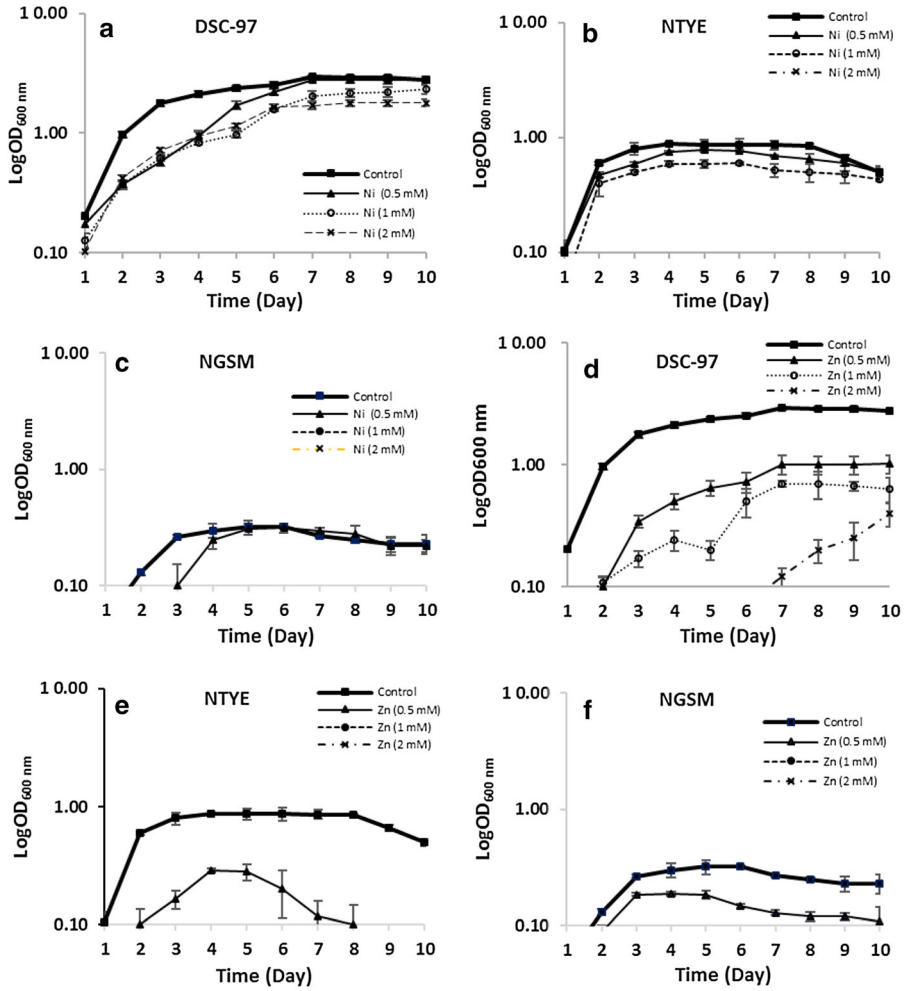

Fig. 3 Effect of nickel and zinc on growth kinetics of *H. salinarum* at different media (**a** and **d**: DSC-97; **b** and **e**: NTYE, **c** and **f**: NGSM)

the other hand, are nonessential. They are not required by microorganisms and are often toxic to cells (Bruins et al. 2000). These heavy metals ions are able to bind to important cellular components through electrostatic interactions or covalent bonding and inactivate their functions.

The computed μmax and generation time for the three media used with and without heavy metals were represented in Table 2. The results showed the cultures of *H. salinarum* in DSC-97 (the control) was characterised by an μmax of 1.564 day^{-1} and a generation time of 0.443 days. This later increased significantly (between 0.540 and 2.502 days) with heavy metals at different concentrations. In NTYE, the control was able to grow with an μmax of 1.746 day^{-1} and a generation time of 0.397 days. The generation time increased with all the metals. In NGSM, the control was able to grow with an μmax of 0.708 day^{-1} and a generation time of 0.979 days.

Halobacterium salinarum showed varying resistance in both complex (DSC-97/NTYE) and minimal (NGSM) media with the presence of varying heavy metal concentrations. *H. salinarum* was most resistant in complex media, mainly DSC-97, when compared with the minimal medium. This result was in accordance with the study by Salgaonkar et al. (2016) where they demonstrated resistance may be due to the complex formation by the media ingredients with metals. This complex is able to decrease the availability of metals towards microorganisms. A similar

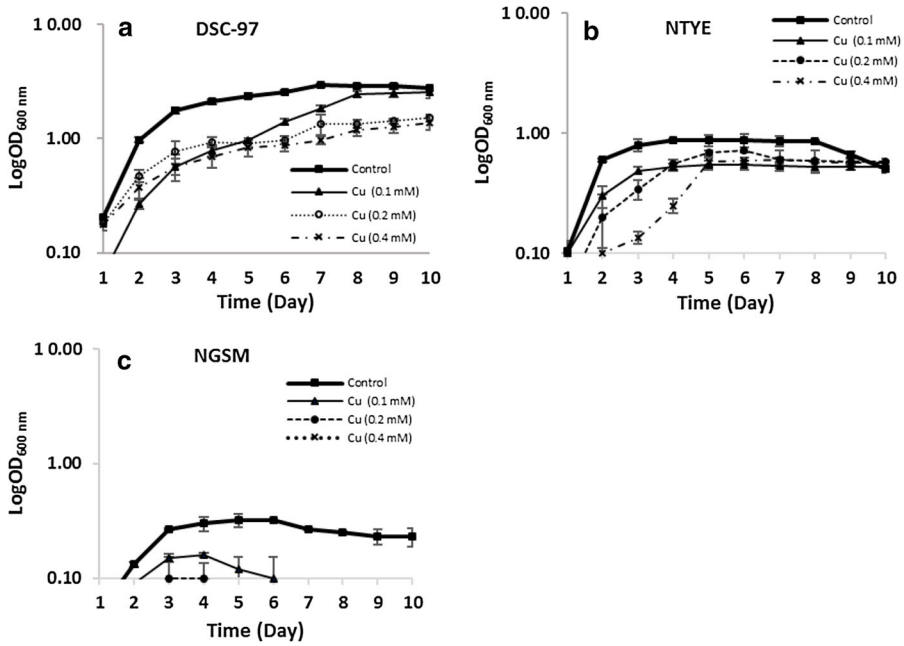

Fig. 4 Effect of copper on growth kinetics of *H. salinarum* at different media (**a** DSC-97, **b** NTYE, and **c** NGSM)

Table 2 Growth rate (μmax) and generation time of *Halobacterium salinarum* grown in DSC-97, complex (NTYE), and minimal (NGSM) media

Growth media	DSC-97		NTYE		NGSM	
	μmax (day^{-1})	t (days)	μmax (day^{-1})	t (days)	μmax (day^{-1})	t (days)
Control	1.564	0.443	1.746	0.397	0.708	0.979
Cd						
0.5 mM	0.702	0.987	1.079	0.642	0.678	1.022
1 mM	0.539	1.286	0.802	0.864	0.665	1.042
2 mM	0.516	1.343	0.467	1.484	0.330	2.100
4 mM	0.287	2.415	–	–	–	–
Pb						
0.5 mM	1.230	0.564	1.680	0.413	0.654	1.060
1 mM	1.283	0.540	0.426	1.627	0.619	1.120
2 mM	0.933	0.743	0.393	1.764	–	–
4 mM	0.405	0.711	–	–	–	–
Ni						
0.5 mM	0.510	1.359	1.547	0.448	0.693	1.000
1 mM	0.285	2.432	0.223	3.108	–	–
2 mM	0.277	2.502	–	–	–	–
Zn						
0.5 mM	0.375	1.848	0.500	1.386	0.666	1.041
1 mM	0.349	1.986	–	–	–	–
Cu						
0.1 mM	0.747	0.928	1.098	0.631	0.510	1.359
0.2 mM	0.943	0.735	0.536	1.293	–	–
0.4 mM	0.432	1.605	0.307	2.258	–	–

μmax Maximum growth rate, *t* generation time

observation was made by Braganca and Furtado (2013) while studying the resistance of the haloarchaeon *Halobacterium* strain R1 to cadmium when grown in minimal medium. In the presence of heavy metals at varying concentrations, the selected strain seemed to be more sensitive in liquid media at concentrations lower than those obtained in solid media. As described previously by Bhojiya and Joshi (2016), heavy metal's toxicity in liquid media is different from toxicity testing on solid medium. The conditions of complexity and availability of metals and diffusion in liquid medium are different from solid medium (Srivastava and Kowshik 2013; Bhojiya and Joshi 2016). For example, Huo et al. (2014) showed *Halomonas zincidurans* B6T resistance to zinc was much higher when incubated on a solid medium than on liquid.

Pigment analyses

When *H. salinarum* was tested in the different media against various concentrations of heavy metals, pigmented cultures ranging from red to very faint orange were observed. The pigments occurring in each culture were extracted and identified based on their spectroscopic characteristics (UV–Vis spectrometry). Pigmentation scans in the 350–650 nm range had varying peaks intensities and exhibited characteristics peaks at 388, 467, 495 and 526, indicative of bacterioruberin pigmentation (Stan-Lotter et al. 2002; Raghavan and Furtado 2005; Wang et al. 2007; Braganca and Furtado 2009; Mani et al. 2012; Salgaonkar et al. 2012). The pigmentation curves for all tested heavy metals are shown in Fig. 1S. The control cultures exhibited the highest absorbance maxima in the absorption spectrum. They showed maximum peak (absorbance of 2.59, 2.18 and 0.32, respectively in DSC-97, NTYE, and NGSM media) at 495 nm. Alterations in pigment production have been previously reported by halophilic *Archaea* under hydrocarbon stress by Raghavan and Furtado (2005). Negative correlations between peak intensities of *H. salinarum* and the metal concentrations were also observed. The culture corresponding with lowest metal concentrations in media exhibited the highest absorbance maxima in the absorption spectrum after the control. In the presence of metal concentrations more than 0.5 mM, a change in pigmentation intensity occurs. This result is in accordance with Chaudhary et al. (2014) who studied the effect of cadmium in the growth of four archaeal strains. They showed the pigment intensity decreased due to either the overall reduced growth of the haloarchaeal culture or the specific interference of heavy metals ions inhibiting the enzymes producing the pigment.

Effect of varying NaCl concentrations and pH

Without heavy metals, *H. salinarum* was able to grow in varying pH (5–9) and NaCl concentrations (200–350 g L^{-1}) with an optimum growth at pH 6 and a salinity of 250 g L^{-1} in the media used above (Fig. 2S). In the presence of 1 mM cadmium (selected as the most tolerated by the strain), the culture grew optimally at pH 5 and a salinity of 250 g L^{-1} (Fig. 2S). At acidic pH, *H. salinarum* can grow with intense pigmentation contrary to the alkaline pH. This result was not in accordance with studies carried out by Das et al. (2014) which showed *Haloferax* strain BBK2 was able to grow in the presence of 1 mM cadmium at a salt concentration range of 50–300 g L^{-1} with an optimum at 250 g L^{-1}. They also reported robust growth at the pH range 7–9 with the best at pH 9. Zouboulis et al. (2004) and Amoozegar et al. (2012) showed the bioavailability of these heavy metal ions is greatly impacted by environmental pH and pH indirectly affects the affinity of these ions to ligand sites on the cell surface. This supports pH being an important variable in metal ion tolerance. Onishi et al. (1984) demonstrated that increasing salt concentration resulted in a decrease of toxicity in cadmium in a *Pseudomonas sp*. strain. A similar trend was reported in *Haloferax* strain BBK2, except for one concentration where the tolerance to cadmium was greatly reduced (Das et al. 2014). Heavy metal ions may interact with the salt ions in the media when there are high salt concentrations, which would allow halotolerant *Archaea* to have increased tolerance to these metal ions when under high salt stress (Nieto et al. 1987; Völkel et al. 2018).

Two-way analysis of variance showed that pH values as well as NaCl concentrations have the same effect on the three media in presence of 1 mM of cadmium (P_{Value} of 0.344 and 0.251, respectively > 0.05), while the media are characterised by different effects on growth (P_{Value} of 0.027 and 0.008, respectively < 0.05). For these media, the computed LSD showed, through multiple comparisons,

significant differences especially between "DSC-97" -NTYE and "DSC-97"-NGSM (Differences $> LSD_{0.05}$).

Conclusion

Halobacterium salinarum was able to tolerate cadmium, lead, nickel, copper, and zinc at different concentrations in complex growth media (DSC-97 and NTYE) as well as in minimal medium (NGSM) but with less growth density. The growth kinetics in the different media showed *H. salinarum* seemed to be more sensitive in liquid media at concentrations lower than those obtained in solid media. *H. salinarum's* growth in the presence of heavy metals was less than the control and characterised by shorter generation times. Spectrophotometric scans of pigments in selected media were characterised by varying intensities, but lower than in the control. They showed higher concentrations of heavy metals might have an effect on the pigment intensity. In the presence of 1 mM cadmium, chosen as the most tolerated, the culture grew optimally at pH 5 and a salinity of 250 g L^{-1}. At an acidic pH, *H. salinarum* can grow with intense pigmentation contrary to an alkaline pH.

Halobacterium salinarum possesses significant heavy metals tolerance and can further be successfully exploited in biotechnologically for the bioremediation of heavy metals contaminated environments. An in-depth study at the molecular level may help in better understanding the mechanisms involved in its metal tolerance.

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Author's contribution HB isolated the strain, analysed the cultivation data, extracted pigments and wrote the manuscript, MS contributed to text preparation, EA was involved in results evaluation. CD performed the phylogenetic analysis and revised the manuscript, CA and MT conceived the idea, designed the study and supervised HB and MS. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The author declare that they have no conflict of interest.

Human and animal participants This study does not involve any human participants or animal experiments.

References

Al-Momani FA, Massadeh AM, Hadad YA (2007) Uptake of zinc and copper by halophilic bacteria isolated from the Dead Sea Shore, Jordan. Biol Trace Elem Res 115:291–300

Amoozegar MA, Hamedi J, Dadashipour M, Shariatpanahi S (2005) Effect of salinity on the tolerance to toxic metals and oxyanions in native moderately halophilic spore-forming bacilli. World J Microbiol Biotechnol 21:1237–1243

Amoozegar MA, Ghazanfar N, Didari M (2012) Lead and cadmium bioremoval by *Halomonas* sp., an exopolysaccharide-producing halophilic bacterium. Progress Biol Scib 2:1–11

Andreini C, Banci L, Bertini I, Rosato A (2006) Zinc through the three domains of life. J Proteome Res 5:3173–3178

Azri C, Maalej A, Medhioub K, Rosset R (2007) Evolution of atmospheric pollutants in the city of Sfax (Tunisia) (October 1996–June 1997). Atmosfera 20:223–246

Azri C, Abida H, Medhioub K (2009) Geochemical behaviour of the Tunisian background aerosols in sirocco wind circulations. Adv Atmos Sci 26:390–402

Azri C, Abida H, Medhioub K (2010) Geochemical behaviour of the aerosol sampled in a suburban zone of Sfax City (Tunisia). Int J Environ Pollut 41:51–69

Baati H, Guermazi S, Amdouni R, Gharsallah N, Sghir A, Ammar E (2008) Prokaryotic diversity of a Tunisian multipond solar saltern. Extremophiles 12:505–517

Baati H, Amdouni R, Azri C, Gharsallah N, Ammar E (2012) Brines modelling progress: a management tool for Tunisian multipond solar salterns, based on physical, chemical, and microbial parameters. Geomicrobiol J 29:139–150

Baati H, Bahloul M, Amdouni R, Azri C (2020) Metal contamination and resistance of superficial sediment's prokaryotic flora in extreme environments: case of Sfax solar saltern (Tunisia). Geomicrobiol J 37:345–354

Bahloul M, Chabbi I, Dammak R, Amdouni R, Medhioub K, Azri C (2015a) Geochemical behaviour of PM10 aerosol constituents under the influence of succeeding anticyclonic/cyclonic situations: case of Sfax City, Southern Tunisia. Environ Monit Assess 177:1–17

Bahloul M, Chabbi I, Sdiri A, Amdouni R, Medhioub K, Azri C (2015b) Spatiotemporal variation of particulate fallout instances in Sfax City, Southern Tunisia: influence of sources and meteorology. Adv Meteorol 471396:11

Berney M, Weilenmann HU, IhssenJ BassinC, Egli T (2006) Specific growth rate determines the sensitivity of

Escherichia coli to thermal, UVA, and solar disinfection. Appl Environ Microbiol 72:2586–2593

Bhojiya AA, Joshi H (2016) Heavy metal tolerance pattern of *Pseudomonas Putida* isolated from heavy metal contaminated soil of Zawar, Udaipur (India). Int J Innov Knowl Concept 2:58–64

Bini E (2010) Archaeal transformation of metals in the environment. FEMS Microbiol Ecol 73:1–16

Bonete MJ, Bautista V, Esclapez J, Bonete MJG, Pire C, Camacho M, Crespo JT, Espinosa RMM (2015) New uses of Haloarchaeal species in bioremediation processes. In: Shiomi N (ed) Advances in bioremediation of wastewater and polluted soil. INTECH, London, pp 23–49

Boujelben I, Martinez-Garcia M, Pelt JV, Maalej S (2014) Diversity of cultivable halophilic *Archaea* and *Bacteria* from superficial hypersaline sediments of Tunisian solar salterns. Antonie Leeuwenhoek 106:675–692

Braganca JM, Furtado I (2009) Isolation and characterization of haloarchaea from low-salinity coastal sediments and waters of Goa. Curr Sci 96:1182–1184

Braganca JM, Furtado I (2013) Resistance of *Halobacterium* strain R1 to cadmium during growth in mineral salts medium devoid of growth factors. Asian J Microbiol Biotech Environ Sci 15:299–302

Bruins MR, Kapil S, Oehme FW (2000) Microbial resistance to metals in the environment. Ecotox Environ Safe 45:198–207

Chasapis CT, Andreini C, Georgiopolou AK, Stefanidou ME, Vlamis-Gardikas A (2017) Identification of the zinc, copper, and cadmium metalloproteome of the protozoon *Tetrahymena thermophila* by systematic bioinformatics. Arch Microbiol 199:1141–1149

Chaudhary A, Pasha IM, Bhakti BS, Braganca JM (2014) Cadmium tolerance by, haloarchaeal strains isolated from solar saltern of Goa, India. Int J Biosci Biochem Bioinforma 4:1–6

Choudhury R, Srivastava S (2001) Zinc resistance mechanisms in bacteria. Curr Sci 81:768–775

Dammak R, Bahloul M, Chabbi I, Azri C (2016) Spatial and temporal variations of dust particle deposition at three urban/suburban areas in Sfax city (Tunisia). Environ Monit Assess 188(6):336

Das D, Salgaonkar BB, Mani K, Braganca JM (2014) Cadmium resistance in extremely halophilic archaeon *Haloferax* strain BBK2. Chemosphere 112:385–392

DasSarma S, Fleischmann EM, Rodriguez-Valera F (1995) Appendix 2. Media for halophiles. In: Robb FT (ed) *Archaea*: a laboratory manual. Cold Spring Harbor Laboratory Press, New York, pp 225–230

DeLong EF (1992) *Archaea* in coastal marine environments. Proc Nalt Acad Sci USA 89:5685–5690

Enache M, Teodosiu G, Faghi AM, Dumitru L (2000) Identification of halophilic Archaebacteria isolated from some Romanian salt lakes on the basis of lipids composition. Rev Roum Biol Ser Biol Veg 45:93–99

Erdogmus SF, Multu B, Korcan SF, Guven K, Konuk M (2013) Aromatic hydrocarbon degradation by halophilic *Archaea* isolated from Camalty saltern, Turkey. Water Air Soil Pollut 224:1449–1458

Gabballa A, Amer R, Hussein H, Moawad H, Sabry S (2003) Heavy metals resistance pattern of moderately halophytic bacteria. Arab J Biotech 6:267–278

Huo YY, Li ZY, Cheng H, Wang CH, Xu XW (2014) High quality draft genome sequence of the heavy metal resistant bacterium *Halomonas zincidurans* type strain B6T. Stand Genomic Sci 9:30–39

Japan International Cooperation Agency "JICA" (1993) The study on waste water treatment and recycling of selected industries in the region of Sfax in the Republic of Tunisia, JICA LIBRARY1115588

Kaur A, Pan M, Meislin M, Facciotti MT, El-Gewely R, Baliga NS (2006) A systems view of haloarchaeal strategies to withstand stress from transition metals. Genome Res 16:841–854

Kottemann M, Kish A, Iloanusi C, Bjork S, Diruggiero J (2005) Physiological responses of the halophilic archaeon *Halobacterium* sp. strain NRC1 to desiccation and gamma irradiation. Extremophiles 9:219–227

Krzmarzick MJ, Taylor DK, Fu X, McCutchan AL (2018) Diversity and niche of *Archaea* in bioremediation. Archaea 1–17

Kumar S, Stecher G, Li M, Knyaz C, Tamur K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549

Lagorce A, Fourçans A, Dutertre M, Bouyssiere B, Zivanovic Y, Confalonieri F (2012) Genome wide transcriptional response of the archaeon *Thermococcus gammatolerans* to cadmium. PLoS ONE 7:35–41

Macomber L, Hausinger RP (2011) Mechanisms of nickel toxicity in microorganisms. Metallomics 3:1153–1162

Mani K, Salgaonkar BB, Braganca JM (2012) Culturable halophilic *Archaea* at the initial and final stages of salt production in a natural solar saltern of Goa, India. Aquat Biosyst 8:15

Moreno ML, Piubeli F, Bonfa MRL, Garcıa MT, Durrant LR, Mellado E (2012) Analysis and characterization of cultivable extremophilic hydrolytic bacterial community in heavy-metal contaminated soils from the Atacama Desert and their biotechnological potentials. J Appl Microbiol 113:550–559

Nieto JJ, Ventosa A, Ruiz-Berraquero F (1987) Susceptibility of halobacteria to heavy metals. Appl Environ Microbiol 53:1199–1202

Onishi H, Kobayashi T, Morita N, Baba M (1984) Effect of salt concentration on the cadmium tolerance of a moderately halophilic cadmium tolerant *Pseudomonas* sp. Agric Biol Chem 48:2441–2448

Oren A (2012) Taxonomy of the family *Halobacteriaceae*: a paradigm for changing concepts in prokaryote systematic. Int J Syst Evol Microbiol 62:263–271

Poli A, Di Donato P, Abbamondi GR, Nicolaus B (2011) Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by *Archaea*. Archaea 1–13

Popescu G, Dumitru L (2009) Biosorption of some heavy metals from media with high salt concentrations by halophilic *Archaea*. Biotechnol Biotechnol Equip 23:791–795

Raghavan TM, Furtado I (2005) Expression of carotenoid pigments of haloarchaeal cultures exposed to aniline. Environ Toxicol 20:165–169

Rios M, Nieto JJ, Ventosa A (1998) Numerical taxonomy of heavy metal-tolerant nonhalophilic bacteria isolated from hypersaline environments. Int Microbiol 1:45–51

Salgaonkar BB, Mani K, Nair A, Gangadharan S, Braganca JM (2012) Interspecific interactions among members of family *Halobacteriaceae* from natural solar salterns. Probiotics Antimicrob Proteins 4:98–107

Salgaonkar BB, Das D, Bragança JM (2016) Resistance of extremely halophilic *Archaea* to zinc and zinc oxide nanoparticles. Appl Nanosci 6:251–258

Shankar AH, Prasad AS (1998) Zinc and immune function: the biological basis of altered resistance to infection. Am J Clin Nutr 68:447–463

Srivastava P, Kowshik M (2013) Mechanisms of metal resistance and homeostasis in haloarchaea. Archaea 1–16

Stan-Lotter H, Pfaffenhuemer M, Legat A, Busse HJ, Radax C, Gruber C (2002) *Halococcus dombrowskii* sp. nov., an archaeal isolate from a permian alpine salt deposit. Int J Syst Evol Microbiol 52:1807–1814

Trigui H, Masmoudi S, Brochier-Armanet C, Barani A, Grégori G, Denis M, Dukan S, Maalej S (2011) Characterization of heterotrophic prokaryote subgroups in the Sfax coastal solar salterns by combining flow cytometry cell sorting and phylogenetic analysis. Extremophiles 15:347–358

Unz RF, Shuttleworth KL (1996) Microbial mobilization and immobilization of heavy metals. Curr Opin Biotechnol 7:307–310

Voica DM, Bartha L, Banciu H, Oren A (2016) Heavy metal resistance in halophilic *Bacteria* and *Archaea*. FEMS Microbiol Lett 363:1–8

Völkel S, Fröls S, Pfeifer F (2018) Heavy metal ion stress on *Halobacterium salinarum* R1 planktonic cells and biofilms. Front Microbiol 9:1–14

Völkel S, Hein S, Benker N, Pfeifer F, Lenz C, Losensky G (2020) How to cope with heavy metal ions: cellular and proteome-level stress response to divalent copper and nickel in *Halobacterium salinarum* R1 planktonic and biofilm cells. Front Microbiol 10:1–15

Wang G, Kennedy SP, Fasiludeen S, Rensing C, DasSarma S (2004) Arsenic resistance in Halobacterium sp. strain NRC-1 examined by using an improved gene knockout system. J Bacteriol 186:3187–3194

Wang QF, Yang WLH, Liu YL, Cao HH, Pfaffenhuemer M, Stan-Lotter H, Guo GQ (2007) *Halococcus qingdaonensis* sp. nov., a halophilic archaeon isolated from a crude sea-salt sample. Int J Syst Evol Microbiol 57:600–604

Williams GP, Gnanadesigan M, Ravikumar S (2013) Biosorption and bio-kinetic properties of solar saltern halobacterial strains for managing Zn^{2+}, As^{2+}, and Cd^{2+} metals. Geomicrobiol J 30:497–500

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 67:1613–1617

Zheng A, Alm EW, Stahl DA, Raskin L (1996) Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl Environ Microbiol 62:4504–4513

Zhuang X, Han Z, Bai Z, Zhuang G, Shim H (2010) Progress in decontamination by halophilic microorganisms in saline wastewater and soil. Environ Pollut 158:1119–1126

Zouboulis AI, Loukidou MX, Matis KA (2004) Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils. Process Biochem 39:909–916