Mem. S.A.It. Vol. 90, 597

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Halophilic bacteria strategies help to understand habitability aspects of the Solar System

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Abstract. Mars and Europa are two of the planetary objects of prime astrobiological importance in the Solar System. The existence of a vast water ocean enriched with sulfates below the icy crust of Europa, with the possibility to find hydrothermal activity as a source of energy, and on the other hand, the discovery of geological evidences pointing to the existence of salty water running on the surface of Mars together with more evidences of an ancient wet Mars, are all facts that have raised the question of whether Europa's interior as well as Mars surface or sub-surface may harbor environments favorable for life subsistence. We performed some systematic studies aimed to investigate the capabilities of *Cobetia marina*, a moderate halophile, and *Bacillus pumilus*, a halotolerant bacterium, to survive to different conditions that mimic the surface of the planet Mars, and the salty water ocean of the satellite Europa. Our results point to a considerable tolerance to salinity displayed by the studied bacteria, even beyond that imposed only by sodium chloride (NaCl). Under these bases, a discussion on the habitability potential of the Europa's ocean where sulfates have been found, and of the surface of Mars, where chlorates and perchlorates have been reported, is presented.

Key words. Europa's ocean – Water on Mars – Planetary habitability – Halophilic bacteria – Astrobiology.

1. Introduction

The search for life beyond Earth ranks the finding of liquid water as the main factor that qualifies a habitable planet or satellite. In this sense, the discovery of evidences pointing to the possibility of water running on ancient (Squyres et al. 2004) or actual Mars (Ojha et al. 2015; Orosei et al. 2018), or the discovery of geological features on the surface of Europa (Pappalardo et al. 1999) supported by magnetometer studies (Khurana et al. 1998) that stated the basis for the existence of an aqueous layer beneath an icy water crust, place these objects in the Solar System as important targets for astrobiological studies.

1.1. Europa's ocean

Spectral evidence from the Near Infrared Mapping Spectrometer (NIMS) has demonstrated that some regions of Europa's surface are incompatible with pure-H_2O ice material (McCord et al. 1998). The chemical nature of the salt proposed to exist in Europa's ocean is quite different to the most abundant salt in terrestrial oceans. While sodium chloride (NaCl) and other chlorides are common in bodies of water on Earth, the spectral evidence shows that sulfates either of magnesium or sodium ($MgSO_4$ or Na_2SO_4) are present on the deep ocean of Europa. The reason can be found after an examination of the material that formed each of these planetary bodies. If Europa was formed from materials similar to a carbonaceous chondrite then models show that the most abundant cations must be Na^+ and Mg^{2+} (Kargel et al. 2000).

In contrast, sodium (Na^+) and chloride (Cl^-) are the main ions in seawater on Earth evidencing differences in origin and evolution: chloride was initially outgassed as HCl along with water in the early time of Earth's history. On the other hand, Na^+ was leached from rocks to make an initial ocean rich in dissolved NaCl (Knauth 1999).

Moreover, Hand & Chyba (2007) constrained limits on the salinity of Europa's ocean based on Galileo magnetometer measurements combined with radio Doppler data-derived interior models and laboratory conductivity *versus* concentration data; such constraints ranged from "freshwater" (i.e., less than 3 g of salt per kg of H_2O) to near-saturation water (around 300 g of salt per kg of H_2O), though their data best fit with a very salty ocean.

Such evidences have raised the question of whether Europa's interior harbors an ocean favorable for life (Pappalardo et al. 1999; Kargel et al. 2000; Marion et al. 2003; Hand & Chyba 2007).

1.2. Water on Mars

Ancient Mars was geologically active, registered an intense volcanic activity (Hartmann et al. 2001) and had water running on its surface according to the discovered signatures indicative of erosion events, huge valleys and canons as well as dry river beds (Carr & Head 2010). More recently, the *in situ* detection of hydrated minerals on igneous rocks, particularly olivine and sulfates of calcium and magnesium, that can only be explained from hydrothermal events, points also to the presence of water (Martínez-Frías et al. 2006; Ling & Wang 2010) on the red planet.

The analysis of the infrared spectral data, obtained by the Compact Reconnaissance Imaging Spectrometer for Mars (CRISM) instrument onboard the Mars Reconnaissance Orbiter (MRO), shows evidence of hydrated salts in locations were recurring slope lineae are most extensive. It has been considered that the source of hydration is the recurring slope lineae activity itself as it can be the result of contemporary water activity on Mars. The observed absorption features are more consistent with magnesium perchlorate, magnesium chlorate and sodium perchlorate (Ojha et al. 2015). The *Planum Australe* region was surveyed between May 2012 and December 2015 using the Mars Advanced Radar for Subsurface and Ionosphere Sounding (MARSIS) instrument on the Mars Express spacecraft and it found evidence of a layer of perchlorate brine trapped below the ice of the South Polar Layer Deposits, showing that liquid water can be stable at depths around 1.5 km (Orosei et al. 2018). Table 1 shows some parameters that describe the main characteristics of Mars, Europa and Earth.

1.3. Halophilic bacteria

As the average salt content on terrestrial oceans is around 3.5% NaCl, all organisms thriving at higher salt concentrations are considered halophiles. However, the stress imposed by salts different from sodium chloride are not necessary the same. In an experimental study on epsotolerance, Crisler and coauthors report the growth of bacterial isolates in culture media with $MgSO_4{\cdot}7H_2O$ and argue a favorable implication for Mars habitability because sulfates are also present in this planet (Crisler et al. 2012). The adaptive strategies for tolerance at high $MgSO_4$ concentrations were not explored, but the authors noticed some differences in the growth rate and stationary-phase maximum densities when their isolates were exposed to different sulfate salts, that were not explained. There are some reports about the substitution of NaCl with other salts (Oren et al. 2014), but very few in the context of exploring the possibilities of survival of terres-

Table 1. Some parameters of the planet Mars and the satellite Europa compared with Earth.

Parameter	Mars	Europa	Earth
Mass (kg)	6.4×10^{23} (0.107 Earths)	4.8×10^{22} (0.008 Earths)	5.9×10^{24}
Equatorial radius (km)	3.4×10^{3} (0.533 Earths)	1.6×10^{3} (0.061 Earths)	6.4×10^{3}
Rotation period (h)	24.623	3.55 (synchronous)	23.934
Surface gravity (g)	0.376	0.134	1
Surface temperature (K)			
Min.	130	50	184
Mean	210	102	288
Max.	308	125	330
Surface pressure (Pa)	6.40×10^{2}	1.0×10^{-7}	1.01×10^{5}
Atmospheric composition (%)	CO_2 (95.3), N_2 (2.7), Ar (1.6), O_2 (0.13), H_2O (0.03)	O_2	N_2 (78.08), O_2 (20.95), Ar (0.93), CO_2 (0.039), H_2O (1, variable)
Liquid water	Brines on the surface, present in the subsurface(?)	Subsurface ocean	Oceans, seas, bays, lakes, rivers
Main elements	C (organics, CO, CO_2, CO_3^{2-}, HCO_3^-), H (H_2, H_2O), N (organics, N_2, NO_2^-, NO, NO_3^-), O ($O^\bullet$, ClO_4^-, oxides), P (PO_4^{3-}), S (SO_4^{2-}, S^{2-}, S)	C (CO_2, CO_3^{2-}, organics), H (H_2O, H_2SO_4), O (H_2O, O2), S (SO_4^{2-})	C (CO_2, CO, HCO_3^-, organics), H (H_2O, H_2), N (NH_3, NO_3^-, N_2, NO_2^-, NO), O (O_2, H_2O, H_2O_2, oxides), P (PO_4^{3-}, ATP, PO_3^{3-}), S (S, $S_2O_3^{2-}$, S_3O_6, H_2S, SO_4^{2-}, S^-)
Energetic processes	Chemolithotrophy, chemoorganotrophy, fermentation (?) depending on the concentration of organics	Chemolithotrophy, chemoorganotrophy	Chemolithotrophy, chemoorganotrophy, anoxygenic photosynthesis

Some data were taken from Cockell et al. (2016)

trial microorganisms in any other extraterrestrial astrobiological important scenario.

One adaptive strategy to osmotic pressure used by halophilic organisms is the synthesis and/or accumulation of organic molecules of low molecular weight and high-water solubility. These molecules are known as compatible solutes as they do not interfere with the metabolism of the organism that incorporate them. Compatible solutes are limited to aid in the stabilization of some enzymes, the maintenance of cell volume and in providing protection from extreme parameters, such as high salinity, high or low temperature, or desiccation. Identified compatible solutes may be classified as amino acids, sugars or polyols, and their derivatives (González-Hernández & Peña 2002; Roberts 2005).

In this paper, we present data on two microorganisms growing under different salt conditions. We compare the growth rate (μ) and the duplication time (t_d) of *Bacillus pumilus* and *Cobetia marina* when exposed to media exhibiting different water activities (a_w) determined by the presence of distinct contents of NaCl, $MgCl_2$, Na_2SO_4, $MgSO_4$, $FeSO_4$, and $CaSO_4$. We characterized the compatible solutes accumulated by the selected strains when submitted to low water activities in liquid cultures by ^1H quantitative Nuclear Magnetic Resonance (qNMR). Our results are discussed in the context of the habitability of Europa's ocean, and Mars' surface.

2. Materials and methods

2.1. The strains and basal media

The non-halophilic *Bacillus pumilus* isolate H3 (GenBank accession number FJ867397) was obtained from a petroleum reservoir production brine located in Ixhuatlán del Sureste (Veracruz, Mexico) and identified by molecular biology techniques (Terrazas 2009; Terrazas et al. 2009). *B. pumilus* was grown in basal medium containing (g/L): 5 peptone, and 3 yeast extract.

The halotolerant *Cobetia marina* DSM 5160 has been isolated from the Great Bay Estuary in New Hampshire, USA (Rosenberg 1983), and was acquired to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). *C. marina* was grown in basal medium containing (g/L): 5.9 $MgCl_2{\cdot}6H_2O$, 1.8 $CaCl_2{\cdot}2H_2O$, 1 KCl, 5 peptone, 0.1 Fe (III) citrate, 3.24 Na_2SO_4, 0.16 Na_2CO_3, 0.08 KBr, 0.034 $SrCl_2$, 0.022 H_3BO_3, 0.0024 NaF, 0.0016 $(NH_4)NO_3$ and 0.008 Na_2HPO_4.

2.2. Strain culture conditions

All culture media were inoculated to have an initial optical density of 0.1 at 630 nm (OD_{630}) in a 50 mL volume. Incubation was performed under constant temperature (30°C for *C. marina*, and 37°C for *B. pumilus*), and oxygenated at 150 rpm. Bacterial growth was monitored as changes in the OD_{630} at regular time intervals using a UV-Vis spectrophotometer until the stationary phase was reached. Specific growth rates (μ) were calculated by performing a linear regression analysis to the linear section of the logarithmic growth curves. The calculated μ values and the corresponding duplication time (t_d) for each experimental condition are shown in Table 2. All incubations and measurements were done by triplicate.

Table 2. Concentration (M), water activity (a_w), growth rate (μ), and duplication time (t_D) for *Cobetia marina* and *Bacillus pumilus* in different modified culture media. N. D. = Not determined.

	M	a_w	μ (h^{-1})	t_d (h)
	Cobetia marina			
NaCl	0.00	0.99	0.21	3.4
	1.37	0.94	0.22	3.2
	2.74	0.89	0.11	6.2
	3.42	0.88	0.06	12.2
	3.76	0.85	0.04	19.2
$MgSO_4$	0.20	0.99	0.19	3.7
	0.41	0.99	0.21	3.4
	0.81	0.98	0.12	5.8
	1.22	0.98	0.08	8.4
Na_2SO_4	0.28	0.99	0.04	15.80
	0.56	0.98	0.05	11.6
	0.84	0.97	0.06	12.2
	0.99	0.96	0.10	6.7
$FeSO_4$	0.004	N. D.	0.16	4.4
	0.007	N. D.	0.17	4.1
$CaSO_4$	0.006	N. D.	0.08	9.3
	0.011	N. D.	0.21	3.3
	Bacillus pumilus			
NaCl	0.00	1.00	0.15	4.5
	0.27	0.99	0.14	4.9
	0.43	0.97	0.88	0.8
	2.01	0.94	0.15	4.7
$MgSO_4$	0.00	1.00	0.15	4.5
	0.34	0.99	0.16	6.0
	0.68	0.97	0.10	6.9
	1.37	0.94	0.02	32.8
Na_2SO_4	0.00	1.00	0.46	1.5
	0.16	0.99	0.41	1.7
	0.98	0.97	0.38	1.8
	0.40	0.94	0.36	1.9
$MgCl_2$	0.00	1.00	0.45	1.6
	0.88	0.99	0.23	3.0
	0.74	0.97	0.22	3.2
	0.32	0.94	0.00	0.0

2.3. Chemicals and reagents

Betaine, ectoine, and hydroxyectoine standards, as well as deuterium oxide (D_2O, 99.9% D-atom), and potassium hydrogen phthalate (KHP) were obtained from Sigma-Aldrich (MO, USA). A phosphate buffered saline (PBS) solution was prepared at the laboratory following standard procedures. Chromabond C_{18} phase was purchase from Machery-Nagel (Düren, Germany).

2.4. Apparatus

Changes in optical density were measured in a UV-Vis spectrophotometer (Agilent 8454) at a wavelength of 630 nm. The a_w values were determined with an AquaLab water activity meter (AquaLab series 3, Decagon, Devices, Inc.). The quantitative nuclear magnetic resonance (qNMR) experiments were carried out on a VARIAN Mercury 400 MHz.

2.5. Determination of water activity

Basal media were supplemented with the corresponding molar concentration of the salts under study (NaCl, $MgCl_2$, Na_2SO_4, $MgSO_4$, $FeSO_4$, or $CaSO_4$) to achieve a particular water activity value (a_w) as shown in Table 2. The resulting culture media have defined values of a_w to facilitate the comparison of the bacterial growth rate (μ) displayed in each case.

2.6. Identification and quantification of compatible solutes

Liquid cultures of *B. pumilus* and *C. marina* were prepared at all concentrations shown in Table 2. Cultures without NaCl were used as control. The compatible solute extraction process was based on the work reported by Roberts (2006).

3. Results and discussion

3.1. Growth of bacterial strains

Bacillus pumilus and *Cobetia marina* grow optimally in their corresponding basal medium depleted of salts where the water activity (a_w) is 1.0. This is not surprising for *B. pumilus*, a non-halophilic bacterium, but it is quite interesting for *C. marina*, a moderate halophilic bacterium, even though this behavior had been reported previously (Rosenberg 1983). Interestingly *B. pumilus* was also able to grow when the basal media was modified with different quantities of NaCl, $MgCl_2$, Na_2SO_4, and $MgSO_4$ as shown in Figure 1a (Figueroa González 2015). It is also noteworthy the behavior displayed by *C. marina*, reporting growth in media modified with $MgSO_4$, Na_2SO_4, $FeSO_4$ and $CaSO_4$ (Figure 1b) (Figueroa González 2015; Rodríguez Pupo 2015).

B. pumilus shows an optimal growth at 0.68 M NaCl. The growth rates (μ) displayed by the cells of this bacterium are slightly higher in $MgSO_4$ than in Na_2SO_4 and $MgCl_2$, within all the interval of a_w values tested. This response has not been reported before probably because halotolerant and halophilic strains are firstly described in terms of their NaCl tolerance. On the other hand, when the culture media was added with $MgCl_2$, the grow rates drastically decrease and are found very close to the a_w value of 0.99. This means that the bacterium is not able to cope with the presence of this salt and no growth is observed at higher concentrations (Figure 1a). These results are noteworthy in different ways. First, it is notable the ability of *B. pumilus*, a non-halophilic bacterium, to effectively deal with the presence of different salts. Then, this strain apparently prefers the culture media enriched with NaCl, and then the media modified with $MgSO_4$ above any other of the tested salts, particularly $MgCl_2$. The highest salt concentrations endured by *B. pumilus* are 0.68 M NaCl, 0.68 M $MgSO_4$, 0.33 M Na_2SO_4, and 0.14 M $MgCl_2$ (Table 2).

The case for *Cobetia marina*, a moderate halophilic bacterium, is quite different (Figure 1b). This strain was also able to grow in all the tested salts. The growth rates in NaCl are higher when compared to the other salts, especially at high a_w. The halophilic character is shown in the wide range of NaCl concentration endured by the strain. There appears to be salt concentrations that display an opti-

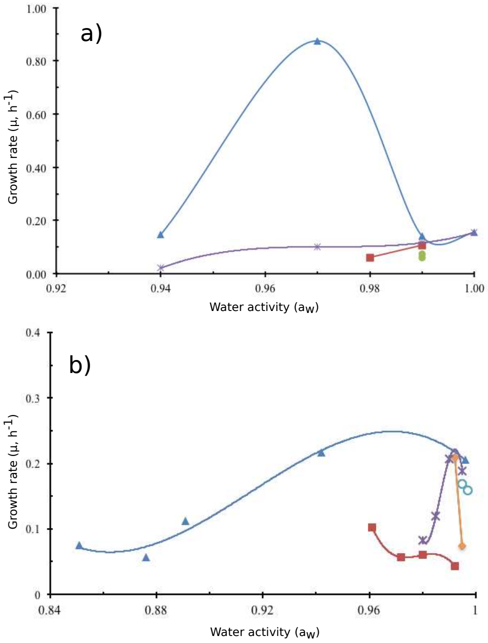

Fig. 1. Specific growth rate (μ) of *B. pumilus* H3 (a), and *C. marina* (b). Different water activities (a_w) due to the presence of NaCl (triangle), $MgCl_2$ (circle), Na_2SO_4 (square), $MgSO_4$ (asterisk), $CaSO_4$ (diamond), and $FeSO_4$ (open circle) were tested. Data points are mean values of three replicates.

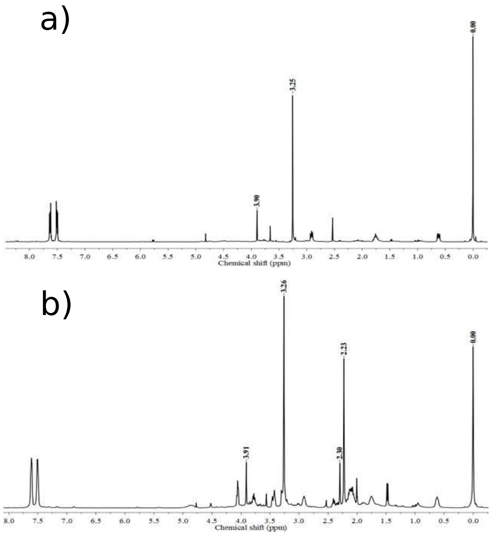

Fig. 2. Nuclear magnetic resonance spectra from cultures of *B. pumilus* H3 with 0.33 M Na_2SO_4 (a), and *C. marina* with 1.37 M NaCl (b). Signals corresponding to the chemical shifts of betaine (3.8, 3.2 ppm), ectoine (2.0 ppm) and hydroxyectoine (4.5, 4.0, 2.3 ppm) are identified. KHP, used as an internal standard, is identified as the signal at 7.6 ppm.

mal growth rate, 1.1 M for NaCl and 0.41 M for $MgSO_4$. It is also noticed that the growth rate in $MgSO_4$ is better than in Na_2SO_4. This is particularly true for a_w values between 0.98 and 0.99. The lowest μ values are displayed in Na_2SO_4. The highest salt concentrations endured by *C. marina* were 3.76 M NaCl, 1.22 M $MgSO_4$, 0.99 M Na_2SO_4, 0.007 M $FeSO_4$, and 0.011 M $CaSO_4$ (Table 2).

3.2. Compatible solute identification and quantification

The NMR spectra corresponding to betaine, ectoine, and hydroxyectoine were used as reference materials for the compatible solutes. The chemical shift, expressed in parts per million (ppm), and the multiplicity of each signal were used as the identification parameters.

The ^1H NMR spectrum obtained at a concentration of 0.33 M Na_2SO_4 for *B. pumilus* shows the two intense signals corresponding to betaine (Figure 2a). On the other hand, the ^1H NMR spectrum for *C. marina* at 1.37 M NaCl shows the signals corresponding to betaine, ectoine and hydroxyectoine (Figure 2b). The signal of all ^1H NMR spectra obtained from the different samples was verified against reference spectra.

When the salt concentration is increased, the intensity of the signals on the ^1H spectra also increased. This is a favorable situation for the identification of the solutes, and also facilitates the quantification of the compatible solute identified at each experimental condition. Thus, it is possible to advance differences in the type of compatible solute accumulated by each bacterium as a function of the saline stress as shown in Table 3.

As far as we know, there is no previous identification of compatible solutes accumu-

Table 3. Concentration (μg of solute per mg of biomass) of compatible solutes accumulated by *Bacillus pumilus* H3 and *Cobetia marina* at different salt concentration. N. I. = Not Identified.

Compatible solute				
	Bacillus pumilus			
	NaCl (M)			
	0.34	1.37		
Betaine	20.30	40.19		
	MgSO4 (M)			
	0.34	1.37		
Betaine	3.11	3.54		
	Cobetia marina			
	NaCl (M)			
	1.37	2.74	3.42	3.76
Betaine	9.74	21.22	12.73	3.44
Ectoine	32.03	10.02	1.06	N. I.
Hydroxyectoine	9.29	13.47	8.52	3.44
	MgSO4 (M)			
	0.20	0.41	0.81	1.22
Betaine	0.43	3.22	1.84	12.238
	Na2SO4 (M)			
	0.28	0.56	0.84	0.99
Betaine	2.98	19.68	23.29	45.19
	CaSO4 (M)			
	0.006	0.011		
Betaine	0.53	0.55		
	FeSO4 (M)			
	0.004	0.007		
Betaine	1.01	1.00		

lated or synthesized by *B pumilus* or by *C. marina* not only in NaCl, but in the other salts used in this study. Different reports corresponding to organisms phylogenetically related have been identified, for example, it has been reported (Kuhlmann & Bremer 2001) that the *Bacillus* genus, without specifying which species, principally synthesize *de novo* glutamate, ectoine and proline. We found ectoine in our experiments. When the salt concentration is increased, betaine seemed to be preferably accumulated. On the other hand, reports identifying ectoine as the main compatible solute synthesized *de novo* by *Halomonas elongata* have been done (Cánovas et al. 1996), but the report is for a minor quantity, the same applied to hydroxyectoine. Our results showed that *C. marina* accumulates betaine and hydroxyectoine at the different NaCl concentrations tested, and the presence of ectoine was found at the lower NaCl concentrations. A report about the production of glutamine and glutamate as compatible solutes when *Halobacillus halophilus* is exposed to 1.0-1.5 M NaCl was done by Saum & Müller (2008). In this same report, it is mentioned that if the salinity increases to 2.0-3.0 M, besides the above solutes, ectoine and proline are also synthesized during culture development. If only the chemical identity of the solutes is considered, we find a better correspondence between our results and those of Saum & Müller (2008). Unfortunately, these authors did not report any quantitative estimation. It should be noted that the presence of glutamate requires confirmation by acquiring its standard spectra as previously mentioned.

4. Conclusions

The search for life in the solar system prioritizes the search for liquid water. This is an outcome derived from the identification of a set of requirements needed for all known of life forms to keep active: a solvent, appropriate temperature conditions, appropriate water activity, and available energy for maintenance, growth or reproduction (Cockell et al. 2016).

An extensive number of studies related to the environment of Europa have pointed to this satellite as a world with the highest potential as a modern habitat for microbial life (Pappalardo et al. 1999; Kargel et al. 2000; Marion et al. 2003; Hand & Chyba 2007; Priscu & Hand 2012) due mainly to the existence of an extensive global ocean that can be geochemically suitable for this kind of life. The temperature of the water in this ocean can be on the order of 253 K, not far from the limit of biological activity on Earth (Neidhardt et al. 1990). Due to the composition of the primordial material proposed for the satellite, sulfur chemistry is important (Priscu & Hand 2012) and then the ocean is probably enriched with sulfates.

Dissolved salts prevent the freezing of the ocean on Europa. Despite this ocean could be about 100 km deep, the pressure in the bottom is not that great because the gravitational acceleration is less than one-seventh of the acceleration on Earth (Priscu & Hand 2012).

It is most likely that if life exists or existed on Europa it would be from the halotolerant, psychrophilic, or barophilic type or a combination of them (i.e., polyextremophile). Here, we have demonstrated that the mesophilic bacterium *Bacillus pumilus* can adequate to saline stress through adaptive strategies such as compatible solute accumulation when its media is modified with NaCl. Moreover, this bacterium can be considered as a halotolerant species due to the fact that was able to grow on NaCl concentrations higher than those found as average on terrestrial water bodies. Besides, *B. pumilus* was also able to cope with the presence of NaCl, $MgCl_2$, Na_2SO_4, $MgSO_4$. We have also found that *Cobetia marina*, a halophilic bacterium, was able to grow not only on cultures modified with NaCl, but also on the presence of $MgCl_2$, Na_2SO_4, $MgSO_4$.

There are no specific values for the salinity on the ocean of Europa. However, empirical constraints have been proposed on the basis of the Galileo data that allow values from 1.1 to 96.8 g of $MgSO_4$ per kg of water (Hand & Chyba 2007). Extrapolating the salt concentration used in our experiments we have covered an interval of 2.4 to 220.3 g of $MgSO_4$ per kg of water. This implies that *Bacillus pumilus* and *Cobetia marina* are perfectly capable of surviving in the actual europan ocean, if just the salinity value is considered. Of course, we have to keep in mind that other constraints should be considered including temperature, pH of the ocean, availability of oxygen, or radiation (Marion et al. 2003). The availability of free-energy is also a critical aspect. In this regard, it has been proposed that metabolisms such as sulfate-reduction, iron reduction, methanogenesis and others that are active in anoxic environments on Earth, might exist on Europa (Gaidos et al. 1999; Priscu & Hand 2012).

On the other hand, sulfates, chlorides, and perchlorates can lower the freezing point of water by up to 80 K, lower that the evaporation rate of water by an order of magnitude, and can be hygroscopic, thus increasing the possibility of forming and stabilizing liquid water on the surface of present Mars (Martín-Torres et al. 2015).

Acknowledgements. This research is supported by grant number 249086, from the AEM-CONACyT program.

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