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Growth of *Bacillus pumilus* and *Halomonas halodurans* in sulfates: prospects for life on Europa

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Abstract

The growth of *Bacillus pumilus* and *Halomonas halodurans* under different concentrations of NaCl, $MgCl_2$, Na_2SO_4 and $MgSO_4$ was investigated. The objective was to demonstrate whether these cultures have the ability to grow, not only in media enriched with sodium chloride, but also with other salts of astrobiological interest. The importance of this monitoring was to evaluate the fitness of these strains to the hypothetical salt content and composition of extraterrestrial sites, such as the ocean of Europa, one of the satellites of Jupiter.

The mechanism of fitness used by these bacteria was investigated by characterizing the compatible solutes accumulated by each strain. *Bacillus pumilus* was cultivated at 0.23 M and 0.33 M NaCl (a_w of 0.995 and 0.990, respectively) while *Halomonas halodurans* was cultivated at 0.44 M and 0.89 M NaCl (a_w of 0.985 and 0.965, respectively). *B. pumilus* seems to accumulate principally betaine while *H. halodurans* accumulates betaine and glutamate, depending on the salt content of its environment. These results are discussed in the context of the salinity and salt composition of Europa's ocean and under their implications for the habitability of this Jovian satellite.

Keywords: Water activity, Europa's habitability, icy satellites, halophiles, compatible solutes.

Resumen

Se presentan los resultados correspondientes al crecimiento de Bacillus pumilus y Halomonas halodurans en distintas concentraciones de NaCl, $MgCl_2$, Na_2SO_4 y $MgSO_4$ para demostrar que estas bacterias tienen la capacidad de desarrollarse en medios enriquecidos, no sólo con cloruro de sodio, sino también con otras sales de interés astrobiológico. La importancia de este estudio radica en evaluar las posibilidades de adecuación de estas bacterias en un escenario hipotético cuyo contenido salino y composición sean parecidos al del océano de Europa, uno de los satélites de Júpiter.

Una manera de evidenciar el mecanismo de adecuación utilizado por estas bacterias es a través de la caracterización química, mediante resonancia magnética nuclear (RMN), de los solutos compatibles acumulados en distintas condiciones de salinidad. Bacillus pumilus se hizo crecer en medios modificados con 0.23 M y 0.33 M de NaCl (a_w = 0.995 y 0.990, respectivamente) mientras que Halomonas halodurans se hizo crecer en medios modificados con 0.44 M y 0.89 M de NaCl (a_w = 0.985 y 0.965, respectivamente). La caracterización de los solutos compatibles demostró que B. pumilus acumula principalmente betaína mientras que H. halodurans acumula betaína y glutamato, según sea la concentración salina de su medio. Se comenta la relevancia de estos resultados en el contexto de la composición salina del océano de Europa y bajo la perspectiva de la potencial habitabilidad de este satélite joviano.

Palabras clave: Actividad de agua, habitabilidad de Europa, satélites helados, bacterias halófilas, solutos compatibles.

1. Introduction

The search for life beyond Earth is focused on the finding of liquid water as the main factor that qualifies a habitable planet or satellite. In this sense, the discovery of geological evidence pointing to the possibility of water running on ancient Mars (Squyres *et al.*, 2004), or the emissions detected on Enceladus as evidence for the existence of internal water pockets (Iess *et al.*, 2014), place some of the objects in the Solar System as important targets for astrobiological studies. One of the most significant results of the Galileo orbiter mission was the discovery of geological features on the surface of Europa (Pappalardo *et al.*, 1999), the smallest of the four Galilean satellites that suggested the existence of an aqueous layer beneath an icy water crust. These observations were supported by magnetometer studies (Khurana *et al.*, 1998). Such evidence 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 and Chyba, 2007).

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). Moreover, Hand and 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 (around 300 g of salt per kg of H_2O), though their data best fit with a very salty ocean.

The chemical nature of the salt proposed to exist in Europa's ocean is quite different from 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 indicating a different 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).

The organisms capable of surviving at high levels of salinity could be halotolerant or halophilic. 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 is not necessarily the same. In an experimental study on *epsotolerance*, Crisler *et al.* (2012) 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 unexplained differences in the growth rate and stationary-phase maximum densities when their isolates were exposed to different sulfate salts. 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 terrestrial microorganisms in an Europan 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 incorporates them. Compatible solutes 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 and 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 *Halomonas halodurans* when exposed to media exhibiting different water activities (a_w) determined by the presence of distinct contents of NaCl, $MgCl_2$, Na_2SO_4, or $MgSO_4$. We characterize the compatible solutes, by ^1H and ^{13}C Nuclear Magnetic Resonance (NMR), accumulated by *B. pumilus* and *H. halodurans* when submitted to low water activities defined by NaCl in liquid cultures. Our results are discussed in the context of the habitability of Europa's ocean.

2. Experimental

2.1. Strains.

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). The halotolerant *Halomonas halodurans* DSM 5160 has been isolated from the Great Bay Estuary in New Hampshire, USA (Rosenberg, 1983), and was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany).

2.2. Determination of water activity in culture media.

Bacillus pumilus isolate H3 grown in basal medium

containing (g/L): 5 peptone, and 3 yeast extract. *Halomonas halodurans* DSM 5160 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. Basal media were supplemented with the corresponding molar concentration of the salts under study (NaCl, $MgCl_2$, Na_2SO_4 or $MgSO_4$) to achieve a particular water activity value (a_w) as shown in Table 1. The resulting culture media have defined values of a_w to facilitate the comparison of the bacterial growth rate (μ) displayed in each case. The a_w values were determined with an AquaLab water activity meter (AquaLab series 3, Decagon, Devices, Inc.).

2.3. Strain culture conditions.

The 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 and agitation (37 ºC, 200 rpm). Bacterial growth was monitored as changes in the OD_{630} at regular time intervals using a microplate reader (Stat Fax 2100) 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 (R^2 values between 88 and 100 % were found). 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.

2.4. Chemicals and reagents.

Betaine and ectoine standards, as well as deuterium oxide (99.9 % D-atom) were obtained from Sigma-Aldrich (MO, USA).

2.5. Apparatus.

All nuclear magnetic resonance (NMR) experiments were carried out on a VARIAN Mercury 400 MHz.

2.6. Extraction of compatible solutes.

Liquid cultures of *B. pumilus* were prepared at 0.23 M and 0.33 M NaCl. For *H. halodurans* the concentrations of 0.44 M and 0.89 M were used. Cultures without NaCl were used as controls. The compatible solute extraction process was based on the work reported by Roberts (2006). Cultures of one-liter were grown until the exponential phase was reached. This means an OD_{630} value of 0.330 for *B. pumilus* and an OD_{630} value of 0.785 for *H. halodurans*. The biomass was separated from the liquid medium by centrifugation at 2486 xg for 20 min. The sediment was washed twice with a NaCl isotonic solution. The sample was dissolved in NaCl isotonic solution and centrifuged again for 15 min. The biomass was suspended in 10 mL of 80 % v/v ethanol (CH_3CH_2OH) and vortexed for 30 min. The mixture was stored for 20 h at 4 °C. After this time, it was centrifuged for 30 min at 3147 xg. The ethanolic supernatant was separated and the ethanol was evaporated using a vacuum chamber. The residue was suspended in 0.5 mL of D_2O and ^1H and ^{13}C spectra were obtained. Resultant spectra are identified as the sample spectra. On the other hand, the spectra of betaine and ectoine were obtained from solutions prepared in D_2O. These were labeled as the standard spectra. The identification of the compatible solutes accumulated by the bacteria was performed by comparing the chemical shift of the signals present on each of the standard spectrum with those present on the sample spectra.

Table 1. Concentration of NaCl, $MgCl_2$, Na_2SO_4 and $MgSO_4$ added to the basal medium of *Bacillus pumilus* H3 and *Halomonas halodurans* and its equivalence to water activity (a_w).

	Molar concentrations (mol/L)			
a_w	**NaCl**	**$MgCl_2$**	**Na_2SO_4**	**$MgSO_4$**
		Bacillus pumilus H3		
1.000	0.00	0.00	0.00	0.00
0.995	0.23	0.2	0.05	0.02
0.990	0.33	0.26	0.22	0.27
0.985	0.43	0.32	0.4	0.56
0.980	0.53	0.38	0.57	0.85
		Halomonas halodurans		
0.991	0.21	0.12	0.01	0.21
0.985	0.44	0.22	0.22	0.62
0.975	0.66	0.38	0.57	1.02
0.965	0.89	0.56	0.93	1.43
0.955	1.12	0.71	1.28	1.83

Table 2. Growth rate (μ) and duplication time (t_d) for *Bacillus pumilus* H3 and *Halomonas halodurans* in culture media modified with different water activities (a_w) as a function of salt concentration.

a_w	**NaCl**		**$MgCl_2$**		**Na_2SO_4**		**$MgSO_4$**	
			Bacillus pumilus H3					
	μ (h^{-1})	t_d (h)	μ (h^{-1})	t_d (h)	μ (h^{-1})	t_d (h)	μ (h^{-1})	t_d (h)
1.000	0.47	1.5	0.45	1.6	0.46	1.5	0.46	1.5
0.995	0.33	2.1	0.23	3	0.41	1.7	0.44	1.6
0.990	0.33	2.1	0.22	3.2	0.38	1.8	0.31	2.2
0.985	0.36	1.9	0.00	0.00	0.36	1.9	0.26	2.7
0.980	0.27	2.6	0.00	0.00	0.35	2.0	0.15	4.6
			Halomonas halodurans					
0.991	0.23	3.0	0.18	3.8	0.2	3.6	0.18	3.8
0.985	0.22	3.2	0.18	4.0	0.25	2.8	0.16	4.3
0.975	0.21	3.4	0.17	4.0	0.26	2.7	0.12	6
0.965	0.04	17.8	0.17	4.1	0.21	3.2	0.08	9.1
0.955	0.04	16.7	0.00	0.00	0.16	4.2	0.02	30.4

3. Results and Discussion

3.1. Growth of bacteria in different salts.

Bacillus pumilus grows optimally in nutritive medium depleted of salts where the water activity (a_w) is 1.0. The cells of this species were 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 1. Interestingly, *B. pumilus* has growth rates (μ) slightly higher when cultured in Na_2SO_4 than in NaCl, 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. Only when a_w values are higher than 0.994 bigger growth rates are displayed in $MgSO_4$ than in NaCl. On the other hand, when the culture media was added with $MgCl_2$, the grow rates drastically decrease until the value of 0.985, when the bacterium is not able to cope with the presence of this salt and no growth is observed (Figure 1). These results are noteworthy in different ways. First, it is notable that *B. pumilus*, a non-halophilic bacterium, has the ability to effectively deal with the presence of different salts. Then, this strain apparently prefers the culture media enriched with Na_2SO_4 above any other of the tested salts, particularly $MgCl_2$. The highest salt concentrations endured by *B. pumilus* are 0.53 M NaCl, 0.26 M $MgCl_2$, 0.57 M Na_2SO_4, and 0.85 M $MgSO_4$ (Table 1).

The case for *Halomonas halodurans*, a moderate halophilic bacterium, is quite different. This strain was also able to grow in all the essayed salts, and again, it seems that the growth rates in Na_2SO_4 are higher when compared with NaCl, $MgCl_2$ and $MgSO_4$ within the interval of a_w values tested. Surprisingly, the growth rates in $MgCl_2$, even when they are slightly lower than in Na_2SO_4, are better than in NaCl and in $MgSO_4$. This is particularly true for a_w values below 0.982. The lowest μ values are displayed in $MgSO_4$. The situation at $a_w = 0.99$ is interesting because it seems that *H. halodurans* prefers NaCl to any of the other salts. There appears to be salt concentrations that display an optimal growth rate, 0.57 M for Na_2SO_4 and 0.38 M for $MgCl_2$. The highest salt concentrations endured by *H. halodurans* were 1.12 M NaCl, 0.56 M $MgCl_2$, 1.28 M Na_2SO_4, and 1.83 M $MgSO_4$ (Table 1).

3.2. Compatible solutes identification.

The NMR spectra corresponding to betaine and ectoine, used as reference materials for the compatible solutes, are shown in Figure 2. The chemical shift, expressed in parts per million (ppm), and the multiplicity of each signal were used as the identification parameters. This information is detailed in Tables 3a and 3b. The NMR spectrum of *B. pumilus* grown in a medium without NaCl is also shown in Figure 2. The ^1H spectrum shows some signals between 1.0 and 4.0 ppm. However, none of them displayed the specific chemical shift of the signals observed on the betaine and the ectoine spectra (Tables 3a and 3b). It can be concluded that there is no presence of any of these compatible solutes in this *B. pumilus* culture. Likewise, the ^{13}C spectrum showed no signals in the range used by the reference materials. *B. pumilus* is a non-halophilic bacterium; consequently, no accumulation of compatible solutes was expected in the absence of salt stress.

The situation is of course different in the cultures modified with NaCl. The ^1H NMR spectrum obtained at a concentration of 0.23 M NaCl ($a_w = 0.995$) showed the two intense signals corresponding to betaine, verified with the help of the reference spectrum (Table 3a). Some other small signals were visible, but due to their low intensity, were difficult to assign. Betaine was also evident in the ^{13}C NMR spectrum, where its three strong signals were visible and could be corroborated likewise with the reference spectrum (Table 3a, and Figure 3). When the NaCl concentration was increased to 0.33 M ($a_w = 0.990$), the intensity of the signals on the ^1H and ^{13}C spectra increased. This was a favorable

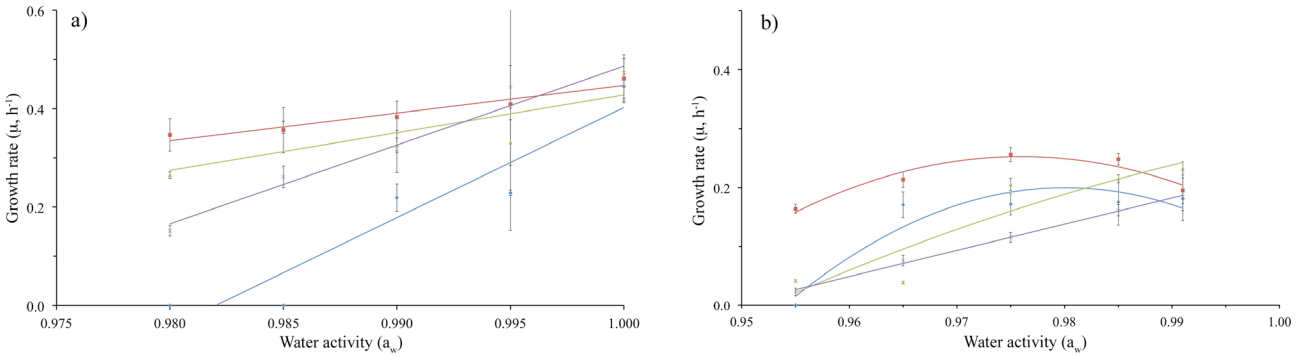

Figure 1. Specific growth rate (μ) of a) *Bacillus pumilus* H3 and b) *Halomonas halodurans*. Different water activities (a_w) due to the presence of NaCl (triangle), $MgCl_2$ (circle), Na_2SO_4 (square), and $MgSO_4$ (asterisk) were tested. Conditions: 50 mL of modified culture medium, incubated at 37 ºC with shaking. Changes in turbidity were measured at 630 nm. Data points are mean values of three replicates.

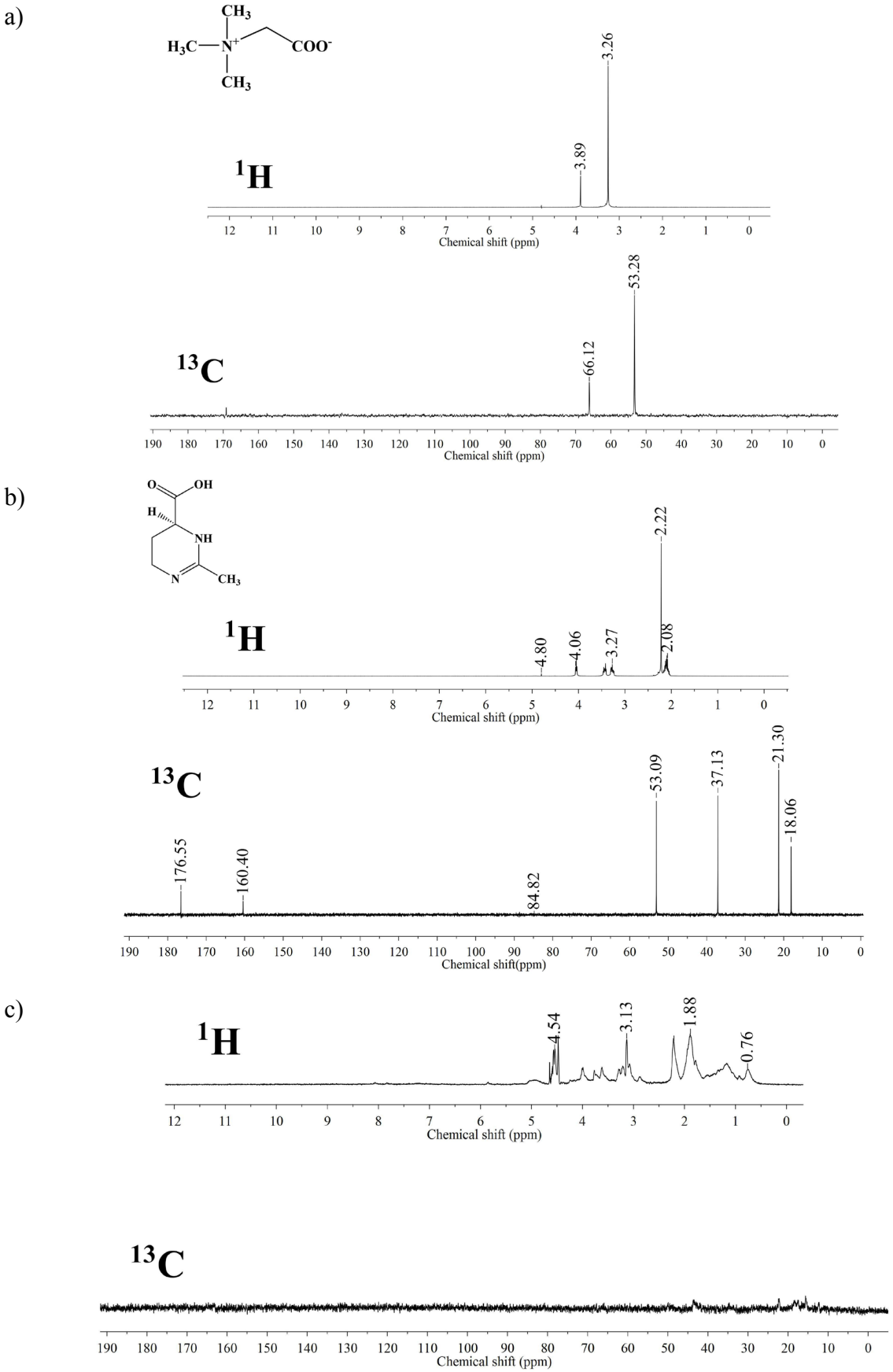

Figure 2. Nuclear magnetic resonance spectra of (*a*) betaine, (*b*) ectoine and (*c*) a *B. pumilus* H3 extract obtained from a culture without NaCl.

Table 3a. Chemical shifts (ppm) and multiplicities of the structural fragments identified in the ^{1}H and ^{13}C NMR spectra corresponding to the compatible solutes present in extracts of *Bacillus pumilus* H3 grown at different NaCl concentrations.

NMR ^{1}H (D_2O, 400 MHz)	Chemical Shift δ(ppm)			NMR ^{13}C (D_2O, 101 MHz)	Chemical Shift δ(ppm)		
	Reference[a]	NaCl (mol/L)			Reference[a]	NaCl (mol/L)	
		0.23	0.33			0.23	0.33
Betaine							
s, 9H, C**H**$_3$	3.08	3.11	3.11	3C, **C**H$_3$	53.23	53.3	53.33
s, 2H, C**H**$_2$	3.71	3.75	3.75	1C, **C**H$_2$	66.01	66.15	66.12
				1C, **C**OO$^-$	169.13	ND	169.21

[a] Roberts, 2006.
ND = no detected signal.

Table 3b. Chemical shifts (ppm) and multiplicities of the structural fragments identified in the ^{1}H and ^{13}C NMR spectra corresponding to the compatible solutes present in extracts of *Halomonas halodurans* grown at different NaCl concentrations.

NMR ^{1}H (D_2O, 400 MHz)	Chemical Shift δ(ppm)			NMR ^{13}C (D_2O, 101 MHz)	Chemical Shift δ(ppm)		
	Reference[a]	NaCl (mol/L)			Reference[a]	NaCl (mol/L)	
		0.23	0.33			0.23	0.33
Betaine							
s, 9H, C**H**$_3$	3.08	3.1	3.12	3C, **C**H$_3$	53.23	53.67	63.77
s, 2H, C**H**$_2$	3.71	3.74	3.76	1C, **C**H$_2$	66.01	66.8	68.76
				1C, **C**OO$^-$	169.13	170	171.9
Ectoine							
m, 2H, C**H**$_2$-CH-COO$^-$	1.89 – 1.97	1.90 – 1.96	1.86 – 1.94	1C, **C**H$_3$	18.03	ND	18.84
s, 3H, C**H**$_3$	2.06	ND	ND	1C, **C**H$_2$-CH-COOO$^-$	21.28	ND	26.39
m, 2H, C**H**$_2$-NH	3.09 – 3.29	3.15 – 3.2	3.20 – 3.29	1C, **C**H$_2$-NH	37.09	ND	35.99
				1C, **C**H-COO$^-$	53.07	ND	55.99
t, 1H, C**H**-COO$^-$	3.89	3.98	3.98	1C, N-**C**=N	160.39	ND	164
				1C, **C**OO$^-$	176.61	ND	177.33

[a] Roberts, 2006.
ND = no detected signal.

situation for the identification of the solutes. The spectra of ^{1}H and ^{13}C NMR for *B. pumilus* at 0.33 M NaCl (a_w = 0.990) are shown in Figure 3. A broad comparison was done, based on the integrated area of the main signal for each identified compatible solute. Thus, it was possible to advance differences in the type of compatible solute accumulated by each bacterium as a function of the concentration of NaCl (Table 4). Additional signals were also present on the ^{13}C NMR spectra and were assigned on the basis of the chemical shifts reported by Roberts (2006) who presented the spectroscopic parameters of some of the most common compatible solutes accumulated by halotolerant and halophilic organisms. In this sense, we find chemical shifts that could be assigned to glutamate. However, these identifications need to be taken cautiously because they must be confirmed through the acquisition and comparison of the spectra of a glutamate standard. Nevertheless, the approach used here can help in the recognition of the compatible solutes used by *B. pumilus*.

The identification of the compatible solutes in the extracts from *H. halodurans* grown in NaCl was a major challenge. The ^{1}H and ^{13}C NMR spectra corresponding to 0.44 M NaCl (a_w = 0.985) showed a larger number of signals. Luckily, some of them could be assigned to the presence of betaine and glutamate, as was shown when compared with the reference spectra and the spectroscopic parameters reported by Roberts (2006) as previously explained.

When the NaCl concentration was increased to 0.89 M (a_w = 0.965), the main signals were attributed to the same solutes. These results are shown in Figure 4. The comparison of the compatible solutes accumulated as a function of NaCl concentration is shown on Table 4.

As far as we know, there is no previous identification of compatible solutes accumulated or synthesized by *Bacillus*

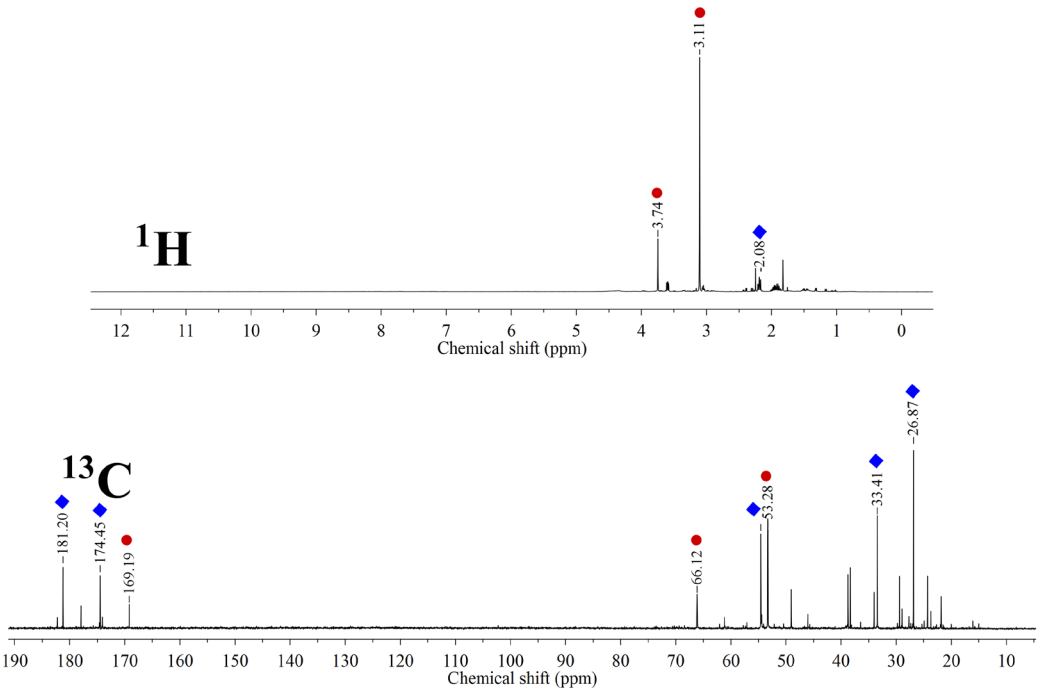

Figure 3. Nuclear magnetic resonance spectra obtained from a *B. pumilus* H3 culture with 0.33 M NaCl. Signals corresponding to the chemical shifts of betaine (red circles), and glutamate (blue diamonds) are identified.

Table 4. Compatible solutes accumulated by *Bacillus pumilus* H3 and *Halomonas halodurans* at different NaCl concentrations.

	Bacillus pumilus H3		*Halomonas halodurans*	
	NaCl (M)			
Compatible solute	0.23	0.33	0.34	0.89
Betaine	++	+++	++	+++
Glutamate[a]	+	+++	+	++

The symbols (+) represent a qualitative appreciation of the quantity of compound identified.
[a] Requires confirmation by comparison with the appropriate standard.

pumilus or by *Halomonas halodurans* in NaCl. Different reports corresponding to organisms phylogenetically related have been identified. For example, Kuhlmann and Bremer (2001) mentioned that the *Bacillus* genus, without specifying which species, principally synthesize *de novo* glutamate, ectoine and proline. We found glutamate in our experiments. When the salt concentration is increased, betaine seemed to be preferably accumulated. On the other hand, Cánovas *et al.* (1996) reported that the main compatible solute synthesized *de novo* by *Halomonas elongata* was ectoine, and in less quantity, also hydroxyectoine. Our results showed that *H. halodurans* accumulates betaine in the two different NaCl concentrations tested, and the presence of glutamate can be suspected at higher NaCl concentrations. Saum and Müller (2008) reported that *Halobacillus halophilus* produces glutamine and glutamate as compatible solutes when exposed to 1.0 – 1.5 M NaCl, but 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 and Müller (2008). Unfortunately, these authors did not report any qualitative or quantitative estimation. It should be noted that the presence of glutamate requires confirmation by acquiring its standard spectra as previously mentioned.

3.3. Implications for the habitability of Europa's ocean.

The search for life in the solar system centers on the search for liquid water mainly due to the fact that life on Earth is defined by three basic requirements: the presence of a sustained source of liquid water, the availability of certain chemical elements to build biomolecules and a source of energy suitable to be used by life. An extensive number of studies, related to the environment of Europa, has 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 and Chyba, 2007; Priscu and 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 and

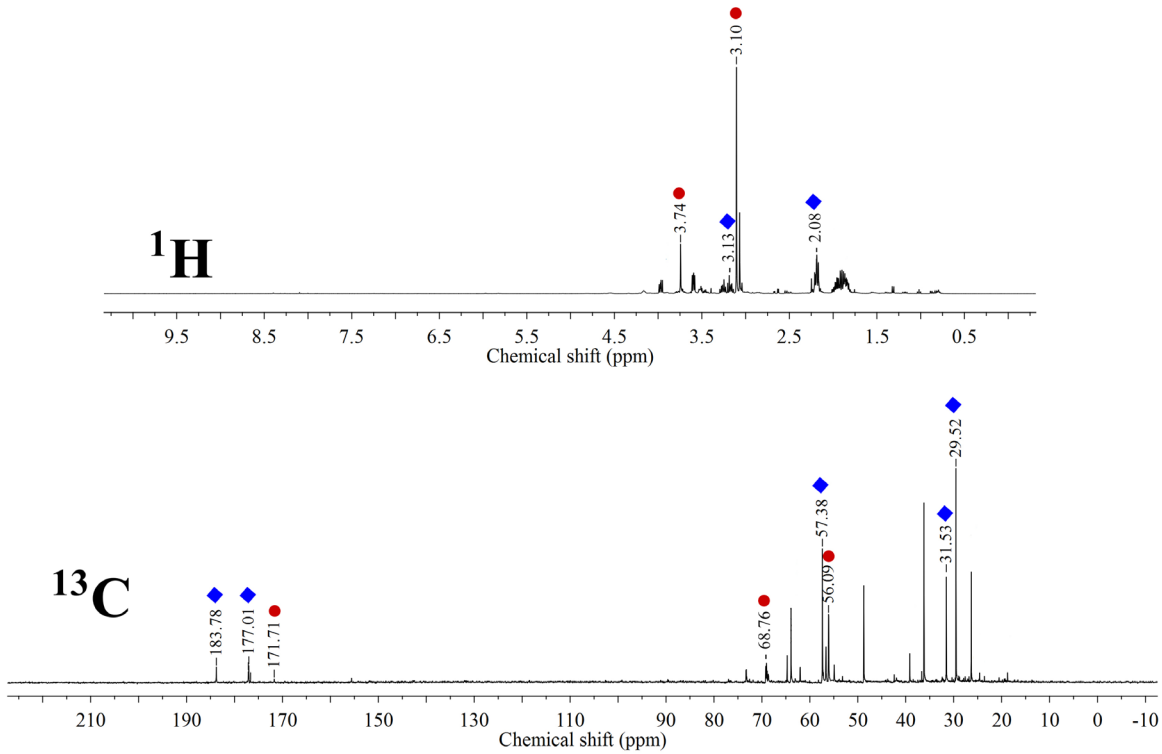

Figure 4. Nuclear magnetic resonance spectra obtained from a *H. halodurans* culture with 0.89 M NaCl. Signals corresponding to the chemical shifts of betaine (red circles), and glutamate (blue diamonds) are identified.

Hand, 2012) as the ocean is probably enriched with sulfates. Dissolved salts prevent the freezing of the ocean on Europa. Despite the Europan ocean's depth, which could be about 100 km, the pressure in its bottom is not that great because the gravitational acceleration is less than one-seventh of the acceleration on Earth (Priscu and 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 adapt to saline stress through strategies such as compatible solute accumulation when its media is modified with NaCl. Moreover, this bacterium can be considered a halotolerant species due to the fact that it 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 and $MgSO_4$. We have also found that *Halomonas halodurans*, 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 and $MgSO_4$. Further studies are needed, in order to determine if a similar strategy, of accumulating compatible solutes, is used when this halophile has to deal with these chemically different salts.

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 and 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 *Halomonas halodurans* 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 and Hand, 2012).

4. Conclusions

We have demonstrated that *Bacillus pumilus*, a non halophilic bacterium, and *Halomonas halodurans*, a moderate halophilic bacterium, were able to adapt to culture media modified with different concentrations of NaCl, $MgCl_2$, Na_2SO_4 and $MgSO_4$ within an interval of water activity (a_w) between 1.0 and 0.98. Information about the strategy used by these bacteria to cope with the stress imposed by the different levels of salinity was inferred by

the identification of some compatible solutes by NMR.

The adaptive strategies used by microorganisms on Earth reveal that most of the physiological stress can be overcome as long as the environment contains liquid water (Ball, 2005). According to the available information of the physical and geochemical parameters for Europa's ocean, and based on our results on the survival of two different bacterial strains at salinity concentrations within the range of the estimated salinity for the Europan ocean, we can infer that this could be a suitable scenario for the presence and persistence of certain forms of terrestrial life.

Detailed studies to define the survival of halophilic bacteria at higher salinity concentrations, as well as the identifications and quantitative estimations on the chemical nature of compatible solutes accumulated on the presence of salts different to NaCl are needed. A mission devoted to perform a detailed analysis of the surface and ocean of Europa to determine the concentration of the salts dissolved in the ocean, and to determine if there are energy sources available for the development of any of the metabolisms known for terrestrial organisms is also needed.

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