Mem. S.A.It. Vol. 90, 658

Memorie della

Poly-extremophiles: exploring the limits of habitability

D. Maizel[1], L. E. Alché[2], and P. J. D. Mauas[1]

[1] Instituto de Astronomía y Física del Espacio, CONICET, UBA, Casilla de Correo 67 - Suc. 28 (C1428ZAA), Buenos Aires, Argentina
[2] Departamento de Química Biológica, CONICET, UBA, 4 Piso, Pabellón II, Ciudad Universitaria (1428), Buenos Aires, Argentina

Abstract. *Brevibacterium linens* AE038-8 is an arsenic hyper-tolerant bacterial strain, previously isolated from well water in Tucumán, Argentina. The aim of this study is to investigate the resistance of this strain to different stress factors relevant for astrobiology studies. We found that *B. linens* AE038-8 is capable of tolerating high concentrations of heavy metals such as Cd(II), Cr(VI) and Cu(II), and a range of temperatures from 10 to 30°C. It is also able to grow in the range of pH values from 3 to 11, and when grown in the presence of NaCl, it can tolerate concentrations up to 5 M. We also study the ability of the strain to grow under combined stress conditions. In particular, we found that it is able to grow under high NaCl concentrations and low pH, conditions relevant to the habitability of brines in Mars.

Key words. Brevibacterium linens – stress factors – poly-extremophiles – astrobiology

1. Introduction

One of the main areas of interest in Astrobiology is the study of extremophiles, organisms that can adapt or even thrive in extreme conditions (Cavicchioli 2002). One of the main reasons for this interest is that the concept of habitability, which refers to the potential of a celestial body to harbor life, is central to Astrobiology. Since the only life forms we know are the ones that can be found on Earth, it becomes fundamental to enlarge our knowledge about extreme life forms inside our own planet, in order to accurately understand which are the physical or geochemical conditions that can limit the presence of life. In particular, life originated on Earth under very extreme conditions!

On the other hand, the study of some types of extremophiles is central to the hypothesis of panspermia, which proposes that certain life forms can survive space travel, and propagate life across different planets. These organisms should be able to survive to high-vacuum conditions, high levels of radiation and extreme temperatures (Abrevaya et al. 2011). These studies are also central to prevent contamination of extraterrestrial environments with terrestrial microorganisms during space missions (Martín-Torres & Zorzano 2017).

Studies of extremophiles cover organisms that can survive in very high concentrations of salt (halophiles) or heavy metals, in very high or low temperatures (thermophiles or psychrophiles), in acid or alkaline environments (acidophiles or alkaliphiles), in extremely dry conditions (xerophiles) or under high level of radiation (radioresistant), among others.

However, conditions in extraterrestrial habitats are most certainly extreme in many aspects at the same time. For example, strong evidence of the existence of salt hydrates and brines on Mars has recently been found (Martín-Torres et al. 2015; Ojha et al. 2015), and it was proposed that these brines could provide a habitable environment (Leuko et al. 2014). Of course, any form of life capable of living in brines should be halophilic, but a high concentration of salt is not the only extreme condition in those environments. Such forms of life should also be able to endure at least low temperatures and high levels of radiation.

Therefore, the next step to understand the real limits of habitability should be to study organisms that can survive under several extreme conditions at the same time, better known as *poly-extremophiles*. In this paper, we study *Brevibacterium linens* AE038-8, a bacterial strain isolated from arsenic-rich groundwater in Tucumán, Argentina. In our previous work, we determined resistance of this strain to arsenicals and to the highest concentrations of arsenic oxyanions reported so far in the literature (Maizel et al. 2016).

Here we investigate the tolerance of *B. linens* AE038-8 to multiple stress factors relevant for astrobiology, such as tolerance to heavy metals, to extreme pH values, different temperatures and to high salt concentrations, conditions which might be present in planets of our Solar System and in exoplanets.

2. Resistance of *B. linens* to different extreme conditions

Temperature: Among the physical parameters important for life, temperature is probably one of the most studied since extreme temperature affects the organisms in different ways, causing destruction of biomolecules (Rothschild & Mancinelli 2001; Prieur 2007). We found that *B. linens* grows at temperatures up to 55°C, and that the optimum growth temperature is 30°C, in accordance with the value reported for other strains of *Brevibacterium* (Sharpe et al. 1977; Collins 2006).

Most extremophiles are mesophiles like this one. Approximately 60% of extremophilic bacteria reported in the literature have temperature optima of 40°C or below: 25% of these bacteria have temperature optima between 38 and 40°C, and approximately 50% between 32 and 37°C, like *B. linens* AE038-8.

pH: Biological processes in normal conditions occur in the middle of the pH spectrum (between 5 and 8.5). Extreme concentrations of hydrogen ions will have a determinant effect on the ability of organisms to thrive in a certain environment (Oarga 2009). The bacterial isolates of the *Brevibacterium* genus reported so far grow at pH values between 3.5 to 8.5 (Lukacs et al. 1995). In this case, *B. linens* AE038-8 shows a great versatility, being able to tolerate pH values from 2.8 to 11. It can therefore be considered as acidophilic, according to the definition of Norris et al. (2000).

Heavy metals: It is well known that many microorganisms able to grow at low pH values not only tolerate, but thrive in metal-rich solutions (Hallberg & Johnson 2001; Johnson et al. 2001). We found that *B. linens* AE038-8 is resistant to high concentrations of Cd(II). In a previous study, we sequenced the complete genome of this strain, and we detected a cadmium resistance operon which might explain this resistance (Maizel et al. 2015). Since bacteria from the Actinobacteria phylum are usually resistant to multiple heavy metals, we studied the resistance to Cr(VI) and Cu(II) as well, and we observed that the bacterial strain is also highly resistant to these metals.

NaCl: The ability of microorganisms to survive in high concentrations of salt is astrobiologically relevant since it has been previously proposed that sodium-chloride rich brines could be present in the surface of Jupiters moon Europa, as a consequence of hydrothermal processes in its seafloor environment (Kargel et al. 2000). We found that *B. linens* can survive in concentrations of NaCl from 0.2 to 5.3 M. This fact means that it is an extreme halophile, according to the conventional classification. In fact, only a few halophiles can survive in the range 3 to 3.5 M NaCl (DasSarma & DasSarma 2017).

Table 1. Growth of B. linens AE038-8 in increasing concentrations of NaCl and NaCl plus arsenic compounds. Growth in each condition was qualitatively determined as +: growth or -: absence of growth.

	NaCl concentration							
	0.2 M	0.5 M	1 M	2 M	3 M	4 M	5 M	5.3 M
NaCl	+	+	+	+	+	+	+	-
NaCl + As(III) 3 mM	+	+	+	+	+	-	-	-
NaCl + As(V) 10 mM	+	+	+	+	+	+	+	-

Many of the extreme halophiles reported so far belong to the Archaea domain. However, some extremely halophilic bacteria have been described, in particular from the phylum Actinobacteria, to which *B. linens* belongs (Bowers et al. 2009). Interestingly, most of the extreme halophiles reported so far were isolated from hypersaline natural environments such as the Great Salt Lake in the United States, the Dead Sea or evaporite sites in the Atacama Desert and Tunisian sabkhas (Stivaletta et al. 2009; Barbieri & Stivaletta 2011; DasSarma & DasSarma 2017). However, the case of *B. linens* AE038-8 is very different, since it was not isolated from a saline environment. Only a few similar cases have been found. For example, Purdy et al. (2004) reported three haloarchaea isolated from estuarine sediments at Essex, UK (with a salinity level similar to that found in sea water), which were able to grow at 2.5% NaCl, a salinity much lower than the one reported here.

Combined effect of NaCl and arsenic: It is important to study the effect of different stress factors combined, since the environments in planets different from Earth will most certainly be extreme in many aspects. We have shown that *B. linens* can tolerate up to the very extreme concentrations of 75 mM and 1 M of As(III) and As(V) respectively (Maizel et al. 2016). Here we investigated its growth in presence of NaCl and arsenic simultaneously. The results are summarized in Table 1.

We found that in a concentration of 3 mM As(III), *B. linens* only grew up to 3 M NaCl, suggesting that both stress factors combined exert a remarkable toxic effect on growth. On the other hand, in a concentration of As(V) 10 mM the strain was able to grow up to 5 M NaCl, similar to what is found in absence of arsenic, showing that arsenate is not significantly toxic for *B. linens*. This result was expected considering that arsenate has been proven to be notably less toxic than arsenite (Hughes 2002).

We also studied the strain's growth-curves in different conditions (Fig. 1). We see that the curve obtained with a concentration 3 mM of As(III) is essentially the same as the control one, but that the presence of salt in a concentration of 2 M introduces a delay in the growth of a 22 hours (lag phase). If arsenite is added, the lag phase remains the same, but a strong difference in the exponential phase can be seen, and growth is 7 times slower. Only after 10 hours both cultures stabilized, reaching the stationary phase with almost the same cell density, noticeably lower than the control one.

Low pH and NaCl combined: It has been suggested that different types of salts in-

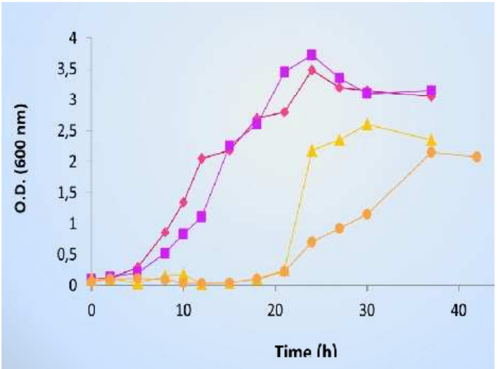

Fig. 1. Bacterial growth under different stressors. Diamonds: control. Squares: As(III) 3mM. Triangles: NaCl 2M; Circles: As(III) 3mM + NaCl 2M

cluding chlorides are abundant on Mars, and that its surface is also acidic (Oren 2014). Although neither liquid water nor brines are currently stable on the red planet, it has been stated that liquid brines could temporarily be present in certain areas as a result of deliquescence (Rennó et al. 2009; Davila et al. 2010). Therefore, microorganisms not only able to tolerate high salinity, but also able to thrive at the lowest end of the pH scale are relevant from an astrobiological perspective. We therefore studied the ability of *B. linens* to grow at pH values of between 2.3 and 4, combined with NaCl concentrations up to 3 M.

At pH 3.5 and 4 growth was observed at 48 h after incubation at all NaCl concentrations tested, suggesting that NaCl does not play a compelling role as a stress factor. With lower pH values of 2.8 and 3, bacterial growth was detected only at NaCl up to 2 M, after 120 h of incubation. The pH value of 2.8 is far lower than the minimum pH reported for growth of strains of the *Brevibacterium* genus. We did not observe any growth at pH 2.3 after 300 h of incubation, even in the absence of NaCl. Since acidic hypersaline environments have been scarcely studied in comparison to prevalent alkaline to neutral hypersaline environments, few halo-tolerant acidophilic microorganisms able to thrive in those conditions have been reported (Seckbach 2013).

3. Discussion

Prokaryotic microorganisms are known to be highly adaptable to different stresses and to thrive in harsh environments. This is probably a consequence of the flexibility of their genomes, which allows life to adapt to a wide spectrum of environmental constraints. As this type of studies evolve, it becomes more evident that resistance to different stressors might be connected. For example, we found that *B. linens* AE038-8 is, on the one hand, tolerant to the highest concentrations of arsenic compounds known so far, and, on the other hand, can grow at high concentrations of heavy metals. This two properties might be related, since it has been shown that an *in vitro* pretreatment of cell cultures with arsenic frequently provides crossed protection against toxicity caused by other metals, such as cadmium (Carrasco et al. 2005).

Metal resistance mechanisms of acidophiles were extensively studied, in particular for copper and cadmium. Dew et al. (1999) reported an example of acidophilic bacteria resistant to toxic concentrations of Cu(II) and Cd(II). Other acidophilic bacteria resistant to Cd(II) were reported by Dopson et al. (2003) and Pikuta et al. (2007).

Additionally, we observe biofilm formation at pH between 3 and 11. The formation of biofilms is generally considered as a cellular defense mechanism from several types of environmental stresses, through physical protection of the bacterial cells and nutrient sequestration (Bollinger et al. 2001; Stewart 2002; Castonguay et al. 2006; Harrison et al. 2007; Koerdt et al. 2010). Thus, the ability of this bacterial strain to form biofilms during growth could partially explain its adaptation to different pH values and temperatures, and the high resistance observed to the many stress factors tested in this study.

In summary, *B. linens* AE038-8 is a bacterial strain isolated from arsenic-rich well water (Maizel et al. 2016). Apart from the arsenic content, the physico-chemical characteristics of this natural environment are not really considered ”extreme”. Even though this strain grows optimally at normal conditions (i.e. 30°C and pH 7), it is able to grow under the many stress factors evaluated in the present work. What makes this bacterial strain highly resistant to different stressors still remains unknown. In this context, we suggest that the habitability range of extremely tolerant bacteria such as *B. linens* AE038-8 is actually much wider than what was assumed until now.

References

Abrevaya, X. C., et al. 2011, Astrobiology, 11, 1034

Barbieri R. & Stivaletta N. 2011, Geol. J. 46, 513

Bollinger, N., et al. 2001, J. Bacteriol., 183, 1990

Bowers, K., et al. 2009, Saline systems, 5, 9

Carrasco, J. A., et al. 2005, Soil. Biol. Biochem., 37, 1131
Castonguay, M. H., et al. 2006, Res. Microbiol., 157, 471
Cavicchioli, R. 2002, Astrobiology, 2, 281
Collins, M. 2006, The genus Brevibacterium. In: The Prokaryotes. Springer, p. 1013
DasSarma, S. & DasSarma, P. 2017, Halophiles. In eLS, John Wiley & Sons, Ltd.
Davila, A. F., et al. 2010, Astrobiology,10, 617
Dew, D. W., et al. 1999, In Alta Copper 99. Brisbane, Australia
Dopson, M., et al. 2003, Microbiol., 149, 1959
Hallberg, K. B. & Johnson, D. B. 2001, Adv. Appl. Microbiol., 49, 37
Harrison, J., et al. 2007, Nat Rev. Microbiol., 5, 928
Hughes, M. F. 2002, Toxicol. Lett., 133, 1
Johnson, D. B., et al. 2001, Environmental Microbiol., 3, 630
Kargel, J. S., et al. 2000, Icarus, 148, 226
Koerdt, A., et al. 2010, PLoS One, 5, e14104
Leuko, S., et al. 2014, Life, 4, 66
Lukacs, A., et al. 1995, Acta dermato-venereologica, 75, 280
Maizel, D., et al. 2015, Genome Announc., 3, e00316
Maizel, D., et al. 2016, International Biodeterioration Biodegradation, 107, 147
Martín-Torres, J., & Zorzano, M.-P. 2017, Astrobiology, 17, 3
Martín-Torres, F. J., et al. 2015, Nature Geoscience, 8, 357
Norris, P. R., et al. 2000, Extremophiles, 4, 71
Oarga, A. 2009, Rev. Biologia e ciencias da Terra, 9, 1
Ojha, L., et al. 2015, Nature Geoscience, 8, 829
Oren, A. 2014, Phil. Trans. Royal Soc. London A, 372, 20140194.
Pikuta, E., et al. 2007, Crit. Rev. Microbiol., 33, 183
Prieur D., 2007, Adv. in Astrob. and Biogeophysics 2, 319
Purdy, K. et al. 2004, Env. Microbiol., 6, 591
Rennó, N. O., et al. 2009, JGR, 114, E00E03
Rothschild, L.J. & Mancinelli, R.L. 2001, Nature, 409, 1092
Sharpe, M. E., et al. 1977, Microbiol., 101, 345
Seckbach, J. 2013, Enigmatic microorganisms and life in extreme environments. Springer
Stewart, P. S. 2002, Int. J. Med. Microbiol., 292, 107
Stivaletta, N., et al. 2009, Planet. Space Sci., 57, 597