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Study of the extremely-tolerant Brevibacterium linens AE038-8 with antiviral activity against Herpes simplex virus type 1

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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 was to characterize this strain regarding its resistance to different stress factors and to evaluate its antiviral activity against Herpes simplex virus type 1 (HSV-1). We found that B. linens AE038-8 was capable of tolerating high concentrations of heavy metals such as Cd(II), Cr(VI) and Cu(II). When grown in the presence of NaCl, it could tolerate up to 3 M in LB25 medium. When cultivated, B. linens released to the supernatants a bioactive principle with antiviral activity against HSV-1 virus regardless growth conditions.
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Response to Reviewers:	Reviewers' Comments to the Authors: Reviewer 1 This manuscript reports the characterization of the resistance of Brevibacterium linens AE038-8, an arsenic hyper-tolerant bacterial strain, to stress factors such as high salt concentrations, heavy metals and high temperature. The ability of a bioactive principle

released by this strain to inhibit herpes simplex type 1 multiplication in cell cultures is also described. This is a concise and interesting study, the experimental approach is adequate and results are conclusive and properly discussed. Though the nature of the antiviral principle remains unknown, the pronounced inhibitory action of bacterial culture supernatants against herpes simplex virus is a remarkable contribution to the discovery of new sources of antiviral agents.
In reference to figures and tables of the manuscript, I think that some modifications in table 4
and figure 2 would make data presentation clearer.

Specific comments
1- B. linens AE038-8 supernatants exert a higher level of CPE inhibition in HSV-1 Field strain infected cells than in HSV-1 KOS infected cultures. What could be the possible explanation to these results? Did other bacterial products exhibit differential efficacy against HSV strains?

Author response: We cannot give an explanation for the higher level of CPE inhibition observed with the supernatants on HSV-1 Field infected cells with respect to HSV-1 KOS infected ones due to the lack of bibliography on this subject related to bacteria. In fact, publications dealing with this issue refer to antivirals of natural origin (plant, algae, fungus-derived, but not bacteria) effective against HSV-1 and HSV-2, but not against TK- strains of HSV (Current Antivirals and Novel Botanical Molecules Interfering With Herpes Simplex Virus Infection, Front Microbiol. 2020; 11: 139).

2- Table 4
In the first column, it would be clearer if instead of a number, the bacteria growing condition is indicated. For instance, instead of 1 it should say LB (Log), instead of 3 it should say LB- NaCl 2 M (Log), and so on. This change would facilitate the interpretation of the results.

Author response: We agree that the new display of the data looks much more clear and easier to interpret. Changes have been made according to the reviewer suggestion. Note: Because of the re-structuration of tables suggested by reviewer #2 as you will note in the comments below, this table is now re-named as Table 2A and Table 2B. Changes have been highlighted in the blinded manuscript under Track Changes.

3- Figure 2
In line with the previous comment, I suggest replacing the numbers by the bacteria growing conditions in the graphics shown in the figure.

Author response: Changes have been made according to the reviewer's suggestion, and numbers were replaced by growing conditions in the Figure.

4- Legend table 4
Please change "cytopathic effect from the virus was evaluated. CV: Viral Control without treatment." To "cytopathic effect from the virus was
evaluated and scored respect to untreated infected control."

Author response: Changes have been made accordingly, and highlighted in the table's text (Please note that this is now Table 2, as mentioned above).

Reviewers' Comments to the Authors: Reviewer 2
This work is based on the study of a bacterial strain of Brevibacterium linens AE038-8 hyper tolerant to arsenic, which was previously isolated from well water in Tucumán, Argentina. The authors focused on characterizing this strain with respect to its resistance to different stress factors, such as salinity and the presence of heavy metals, and evaluated its antiviral capacity against herpes simplex virus type 1 (HSV-1). The experiments carried out are in accordance with the proposed objectives. The study generates novel contributions in the area of knowledge of antiviral agents, particularly against the HSV-1 virus.
Based on the aforementioned, I consider that the manuscript should be accepted for publication in this journal.

	Author response: We appreciate your comment and consideration, as well as your positive feedback. On the other hand, given the limitations of space that the authors have due to the number of pages that are stipulated by this journal, and understanding that it would not be convenient to remove the information presented, I suggest that the results reported in Tables 1, 2 and 3 could be condensed into a single table. Author response: We have condensed tables 1, 2 and 3 into a single one, which is now renamed as Table 1. Note: table's numbers have been changed accordingly and highlighted in the blinded manuscript under track changes.

Study of the extremely-tolerant *Brevibacterium linens* AE038-8 with antiviral activity against Herpes simplex virus type 1

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Running title: An extremely-tolerant bacterium with antiherpetic activity

Aknowledgments

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Author Disclosure Statement.

No competing financial interests exist.

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 was to characterize this strain regarding its resistance to different stress factors and to evaluate its antiviral activity against Herpes simplex virus type 1 (HSV-1). We found that *B. linens* AE038-8 was capable of tolerating high concentrations of heavy metals such as Cd(II), Cr(VI) and Cu(II). When grown in the presence of NaCl, it could tolerate up to 3 M in LB_{25} medium. When cultivated, *B. linens* released to the supernatants a bioactive principle with antiviral activity against HSV-1 virus regardless growth conditions.

Keywords: *B. linens*- halo-tolerant- antiviral activity - Herpes simplex virus

1. Introduction

Extreme environments often constitute an interesting source of microorganisms with the ability not only to overcome but to thrive in those extreme conditions [14]. Many of these extreme microorganisms (also known as extremophiles) produce unexploited and promising bioactive molecules for biotechnological and pharmaceutical applications [9, 13, 26, 27]. These molecules consist of secondary metabolites often produced during growth as a response mechanism to unusual environmental conditions, and they have potential implications in drug discovery [7, 16, 32]. Herpes simplex virus (HSV) infections are quite common in humans. HSV is involved in facial infections, many ocular diseases such as stromal keratitis, encephalitis and neurotrophic keratopathy [14]. More than 80% of humans worldwide are infected with HSV-1, and roughly 40% have recurrent infections [12].

Since HSV infections cannot be managed by vaccination, treatment is currently based on the use of several selective drugs such as acyclovir (ACV), penciclovir, famciclovir, cidofovir, valacyclovir, trifluridine and vidarabine [8, 15, 34]. The intensive clinical use mainly due to reactivation of latent HSV or even the abuse of these agents has led to the emergence of drug-resistant strains [19]. Therefore, there is a an urgent need of new antiherpetic drugs so as to deal with those HSV populations resistant to ACV- the treatment of choice in herpetic infections - and to improve the efficacy and pharmacokinetics of actual treatments, especially in inmunocompromised hosts [17].

We have studied *Brevibacterium linens* AE038-8, and we found that it can resist the highest concentrations of arsenic oxyanions reported so far in the literature for bacteria classified as arsenic-resistant [21].

Although secondary metabolites produced by bacteria have been developed to treat a variety of diseases, to date there has been limited investigation of this kind of products with antiviral activity.

Hence, the aim of this study was to characterize *Brevibacterium linens* AE038-8 in terms of its resistance to different stress factors other than arsenic and to evaluate the production of bioactive principles with antiherpetic activity when grown *in vitro* under the effect of these stressors.

2. Materials and Methods

2.1. Chemicals, culture media and bacterial strain

Arsenite (As[III]) was added as Na_2AsO_2 (>99% purity). Reagents were purchased from Fluka Analytical (Sigma Aldrich Co., St. Louis, MO, US). All chemicals used in this study were analytical grade or better. Stock solutions used in all experiments were prepared at the concentration of 0.01 M.

The bacterial strain used in this study was *Brevibacterium linens* AE038-8 (BNM 562), isolated from arsenic-rich well water from the Tucuman province, Argentina, and previously reported regarding its hyper-tolerance to arsenic compounds [21].

2.2. Characterization of *B. linens* AE038-8 regarding multiple resistances

An inoculum of the bacterial strain (100 ml) was grown overnight on LB_{25} broth [21] at 30°C on a rotary shaker at 150 rpm.

Each experiment was conducted in flasks of 125 ml capacity with 20 ml of LB_{25}, except for the evaluation of tolerance to inorganic arsenic and to NaCl, for which reaction tubes containing 5 ml of LB_{25} as final volume were used. Each assay was performed by triplicate.

2.3. Growth at different temperatures

Flasks were inoculated with the pre-inoculum of the bacterial strain at 10% of the final volume, and were incubated 24 h at the temperatures of 10, 20, 30 and 55° C.

2.4. Tolerance to heavy metals

Tolerance of *B. linens* AE038-8 to Cu(II) (2 and 4 mM), Cr(VI) (1 and 2 mM) and Cd(II) (0.5 and 1 mM) was assayed in LB_{25} agar plates at pH 7.0.

Plates amended with various concentrations of heavy metals were streaked with the strain [29]. After 48 h of incubation, tolerance to the corresponding metal was determined semi-quantitatively by measuring length of the bacterial growth along the streaking line.

2.5. Resistance to NaCl and NaCl plus arsenic

Tubes containing 5 ml of LB_{25} as final volume were amended with plain NaCl in final concentrations of 0.5, 2 and 3 M. A pre-inoculum of the bacterial strain was prepared as previously described in section 2.2, and tubes (including LB_{25} control) were inoculated with 3 x 10^7 Colonies Forming Units per mililiter ($CFU.ml^{-1}$). Tubes were incubated 48 h at 30°C and permanent shaking at 150 rpm until the observation of growth. Additionally, tubes with LB_{25} amended with each of the mentioned NaCl concentrations and without bacterial inoculum were used as control, in order to assure no NaCl precipitation. The same procedure was applied in order to study bacterial growth under NaCl plus arsenite salt, for which arsenite was added in a final concentration of 3 mM to tubes containing LB_{25} amended with NaCl in the above mentioned concentrations. Incubation conditions were the same than those described for the other experiments. Growth after the incubation period in each condition was determined by counting of $CFU.ml^{-1}$, compared with the respective control.

2.6. Growth curves of *B. linens* AE038-8 under the effect of NaCl and NaCl plus arsenic as stressors

Growth was carried out in flasks containing 100 ml of culture medium under the following conditions: LB_{25} (control), LB_{25} + 3 mM As(III), LB_{25} + 2 M NaCl, and LB_{25} + As(III) 3 mM + NaCl 2 M. A pre-inoculum of the strain was prepared as previously described. The inoculum was standardized to an optical density (600 nm) of 0.1 for each flask. Incubation was carried out at 30°C and permanent shaking at 150 rpm for 42 h. One ml aliquots were taken out of each flask every 2 h, and optical density (600 nm) was measured to quantify bacterial growth.

2.7. Cells and viruses

Vero cells were grown in minimal essential medium (MEM) supplemented with 5% inactivated calf serum and 50 µg/ml gentamycin, and maintained in monolayer formation in MEM supplemented with 1.5% inactivated calf serum.

HSV-1 KOS strain (wt, TK+ sensitive to ACV)) and Field strain (TK-, resistant to ACV) were propagated at low multiplicity of infection (m.o.i.), plaque-assayed on Vero cell monolayers, and used for *in vitro* experiments.

2.8. Antiviral screening

Vero cells grown in 96-well plates were infected with HSV-1 at a m.o.i of 0.07 and, after virus adsorption, cells were treated or not with serial dilutions of bacterial supernatants, in a range where no cytotoxicity was observed. After 24 h of incubation at 37º C in 5% CO_2 atmosphere, cytopathic effect (CPE) was evaluated under microscope and scored as a percentage with respect to untreated infected control (1: 25%, 2: 50%, 3: 75%, 4: 100% CPE). Supernatants were harvested and quantified for viral load.

2.9. Viral titration

Vero cells grown in 24-well plates were infected with serial 10-fold dilutions of viral yields and incubated for 1 h at 37º C. Residual inocula were replaced by MEM 2x mixed with 1.4% methylcellulose. After 72 h of incubation at 37º C, cells were fixed with formaldehyde 10%, stained with Crystal Violet, and plaque forming units were counted.

2.10. Approach to characterize the antiviral principle

Three hundred µl of cell-free supernatants, as well as 300 µl of uncultivated medium were concentrated by lyophilization and the resulting powder was resuspended in 50 ul of distilled water. Both solutions were analyzed by means of a TLC (silica gel 60 F254 -Merck). The chromatography was developed using methanol: 1: 2 chloroform. For the detection of peptide moieties 0.5% ninhydrin in acetone was used, for glycosidic moieties Molicsh

reagent was used. Unspecific organic compounds were detected by UV exposition.

3. Results

3.1. Growth at different temperatures

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 [30, 31].

Thus, we have investigated growth of *B. linens* AE038-8 at different temperatures. *B. linens* was able to grow at the different temperatures evaluated, except for the highest one of 55°C (Table 1), in accordance with the corresponding values reported for other strains of *Brevibacterium* [5, 20, 35].

3.2. Resistance to heavy metals

It is well known that many microorganisms are able to thrive in metal-rich solutions [11]. When growth of *B. linens* AE038-8 was tested at different concentrations of Cd(II), we found that it showed high resistance to this metal, which might be explained by the presence of a previously found cadmium operon [21]. Given that bacteria from the Phylum Actinobacteria are usually resistant to multiple heavy metals, we assayed resistance to Cr(VI) and Cu(II) as well, and observed that *B. linens* AE038-8 was also highly resistant to these metals (Table 1).

3.3. Combined effect of salts in bacterial growth

Besides resistance to heavy-metals, many microorganisms typically share other properties such as resistance to high concentrations of salt when growth takes place in extremophilic habitats [24]. *B. linens* AE038-8 was grown in LB_{25} amended with increasing concentrations of NaCl, from 0.5 to 3 M. Growth was observed at almost all the concentrations tested after 48 h of incubation (Table 1). It is well known that stress factors rarely occur individually in extreme environments. Thus, we investigated *B. linens* AE038-8 growth in presence of both NaCl and arsenic.

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We have previously shown that *B. linens* AE038-8 can tolerate up to 75 mM and 1 M of As(III) and As(V) respectively, concentrations that can be considered extreme according to the literature [21]. To study the combined effect of NaCl and arsenic on *B. linens* AE038-8, we assayed a lower concentration of As(III) (3 mM) enough to induce selective pressure on bacterial growth while achieving a relatively fast growth rate. When LB_{25} medium was amended with increasing concentrations of NaCl + 3 mM As(III), *B. linens* AE038-8 could grow up to 3 M NaCl (Table ~~3~~1).

Based on the different levels of resistance observed when *B. linens* AE038-8 was grown with NaCl alone and with NaCl + As(III) (Table ~~3~~1), we investigated the strain's growth profile in each condition (Fig. 1).

For this purpose, we utilized 2 M NaCl, a concentration high enough to exert a considerable stress on the bacterial cells while allowing a relatively rapid growth. When grown in plain LB_{25} medium (control), bacterial growth was relatively fast, with a very short lag phase (2 h), as reported in our previous work [21], and a log phase of 23 h was observed.

When As(III) was amendedF to LB_{25} medium, the bacterial growth was almost identical to that observed in the absence of the metalloid, indicating that arsenite does not play a significant role as a stress factor in the concentration used in our study. This can be explained considering that *B. linens* AE038-8 is already adapted to the selective pressure caused by arsenic, since it was isolated from arsenic-rich waters and, additionally, it was demonstrated that it can tolerate up to a concentration of 75 mM As(III) [21].

When grown in presence of 2 M NaCl and 2 M NaCl + 3 mM As(III), we observed a lag phase of 21 h for the cell culture in both conditions. Considering that the bacterial population was subjected to an appreciable stress, a remarkably long lag phase was expected compared to bacterial growth in plain LB_{25} and LB_{25} + As(III), where after 23 h of incubation the culture reached the stationary phase. Thus, *B. linens* AE038-8 entered the exponential phase under both growth conditions at the same time. Nevertheless, an

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important difference between both log phases was observed. Growth in the presence of NaCl 2 M was 6.8 times faster than growth with 2 M NaCl + 3 mM arsenite, in contrast to that observed for LB_{25} control and LB_{25} plus arsenite (where log phases were almost identical) showing an enhancing effect of both stressors combined. After 37 h of incubation, both cultures stabilized and reached the stationary phase achieving almost the same cell density.

3.4. Screening of antiviral activity in supernatants from *B. linens*

Extreme environments offer an almost unexplored source of microorganisms producing unexploited and promising biomolecules for pharmaceutical applications [9]. Kim *et al.* isolated xiamycins from a *Streptomyces* sp. (#HK18) belonging to a Korean solar saltern with inhibitory activity on porcine epidemic diarrhea virus [18].

Since salt requiring microbes are a robust source of new natural products serve as model systems in drug discovery [2] and *B. linens* AE038-8 proved to be resistant to NaCl, we investigated its ability to produce an antiviral principle against HSV-1. At 23 h and 37 h of incubation at 30°C, all supernatants were harvested, centrifuged at 112 *g* and dialyzed against bidistillated water for biological evaluation (Fig. 2). First, to perform a qualitative screening, each supernatant was two-fold diluted in MEM 1.5% and its capacity to inhibit the CPE caused by HSV-1 infection in Vero cells was evaluated. For that purpose, Vero cells grown in 96-well plates were infected as described in M and M. At 24 h post-infection (p.i.) with HSV-1 KOS strain, ½ dilution belonging to all supernatants reduced viral CPE to 25-50% with respect to untreated infected control cells. This protective effect was lost at 1/4 dilution, in which treated infected monolayers showed a CPE comparable to that of untreated infected cells (Table ~~4~~2A).

When Vero cells were infected with HSV-1 Field strain, a stronger protection of all supernatants assayed was registered since a considerable reduction of CPE was observed in a range of ½ to 1/8 supernatant dilutions (Table ~~4B~~2B). The 96-well plates infected with both strains of HSV-1 were

frozen at -80°C and thawed once for further supernatant collection and viral titration.

3.5. Quantification of HSV-1 yields

To evaluate whether the protective effect of *B. linens* supernatants observed through CPE inhibition correlated with a reduction in HSV-1 yields, supernatants from control and treated HSV-1-infected cells were harvested for viral titration, starting from those with a CPE score between 1 and 2 to those corresponding to a CPE degree comparable to that of untreated infected cells.

Since 1/2-1/16 dilutions of *B. linens* supernatants exerted an inhibitory effect on the multiplication of the Field strain of HSV-1 while only the ½ dilution of the same supernatants could restrain HSV-1 KOS strain propagation, we decided to quantify all the viral yields belonging to infection with the Field strain. In this case, a dose-response pattern of inhibition can also be observed, together with the fact that this type of HSV-1 mutants requires new antiviral principles for its treatment (Table ~~4~~2).

Thus, supernatants belonging to Vero cells infected with HSV-1 Field strain previously treated with supernatants from *B. linens* grown or not with As(III), NaCl or As(III)+NaCl (Table ~~4B~~2B), were titrated in Vero cells grown in 24-well plates.

A significant inhibition of HSV-1 multiplication was observed, regardless the moment of supernatant harvesting (*B. linens* log or stationary phase of growth) (Fig. 2). In all cases, a drop of two-three logs in viral yields with respect to untreated control virus was found, no matter what conditions of *B. linens* growth have been tested (Fig. 2).

Hence, *B. linens* released a bioactive principle with significant antiviral activity against the Field strain of HSV-1, in the presence or absence of selective pressure of any of the salts assayed.

3.6. Approach to the characterization of the antiviral compound

Cell free supernatant presented only one principal spot with a FR=0.243 when observing the TLC under uv light (254nm) while the

uninoculated media presented 4 different spots with FR= 0.271, 0.4, 0.671 and 0.9 respectively. The 0.243 spot showed no reaction neither with ninhydrin nor Molisch reagent suggesting that this compound secreted by *B. linens* AE038-8 has neither glycosidic nor peptidic moieties.

Further studies are needed to characterize the antiviral principle.

4. Discussion

Prokaryotic microorganisms are known to be highly adaptable to diverse environmental stress conditions and to thrive in harsh environments, as a consequence of the flexibility of their genomes, which allowed life to adapt to a wide spectrum of environmental constraints [28].

Based on the results obtained, *B. linens* AE038-8 turned out to be a very interesting subject of study due to its remarkable resistance to salinity as well as to the other stressors we investigated.

In relation to the temperature range tested, *B. linens* grew at 20ºC, temperature registered for the water from which it was isolated [21], in accordance with the optimum growth temperature for other *Brevibacterium* strains [5]. Therefore, *B. linens* AE038-8 is a mesophile as most extreme halophiles [3].

B. linens AE038-8 was also able to grow at the different concentrations of heavy metals tested, showing a high tolerance level (Table ~~2~~1). It is worth noting that resistance to arsenic compounds and to heavy metals is probably connected. It has been previously shown that an *in vitro* pre-treatment of the cell culture with arsenic can frequently provide crossed protection against toxicity caused by other metals like cadmium [4]. On the other hand, high concentrations of chromium have also been previously determined in superficial and ground waters of Los Pereyra [23], which could explain the resistance observed by the bacterial strain.

Many of the extreme halophiles and halo-tolerant microorganisms reported so far belong to the Archaea domain. However, some extremely

halophilic bacteria have been described, such as *Salinibacter ruber*, *Halorhodospira halochloris* and *Natranaerobius grantii* and bacteria from the Phylum Actinobacteria, to which *B. linens* AE038-8 belongs, among others [3].

For example, halophilic bacteria tolerate and grow in the presence of salt concentrations 10 times higher than seawater [36], in environments where water availability is greatly reduced by salt [6, 25].

When bacterial growth was carried out at different concentrations of NaCl, *B. linens* AE038-8 grew up to the concentration of 3 M (Table ~~3~~1). Given its tolerance to NaCl it can be considered as an extreme halo-tolerant bacterium even though it was isolated from a non-extremely saline environment. In fact, this condition was corroborated by means of *B. linens* growth curve in the presence of NaCl 2M, which showed an extended lag phase and a diminished growth rate, confirming its halo-tolerant condition (Fig. 1).

Other halo-tolerant bacterial strains from the Phylum Actinobacteria with similar resistance levels to salt were reported by Seck *et al*., although such strains were isolated from human gut [33].

Secondary metabolites produced by actinobacteria have been a valuable source for the discovery of new bioactive compounds and its characterization has yielded many important drug leads. The emerging threat of drug-resistant microbes, particularly HSV-1 mutants resistant to ACV, makes it crucial to produce novel and unique drugs, possibly from new sources of actinobacteria, such as *B. linens* [37]. Furthermore, anti-HSV-1 activity of another actinobacteria, *Bifidobacterium adolescentis* SPM 0214, was also reported [1]. In our case, *B. linens* released a bioactive principle with antiherpetic activity in any of the growth conditions assayed, that is, with or without selective pressure of NaCl, As(III) or both combined (Table ~~4~~ 2 and Fig. 2). Furthermore, the principle with anti-HSV-1 activity yielded by *B.*

linens seemed not to be related with the number of bacteria measured for each growth condition (Fig. 1).

Therefore, *B. linens* AE038-8 secretes a bioactive principle with proven antiviral activity against the multiplication of a mutant strain of HSV-1 resistant to ACV, which hardly responds to any other conventional treatment. In order to contribute with new antiviral drugs effective against HSV-1 populations resistant to antivirals currently in use, further studies to characterize the compound/s responsible for this bioactivity should therefore be continued.

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B. linens AE038-8			
Temperature (°C)		10	+
		20	+
		30	+
		55	-
Tolerance to heavy metals	Cr(VI) (mM)	1	+++
		2	+++
	Cu(II) (mM)	2	+++
		4	++
	Cd(II) (mM)	0.5	++
		1	++
Addition of salts	NaCl	0 M (LB25 control)	3.5×10^{11}
		0.5 M	3.2×10^{11}
		2 M	3.3×10^{11}
		3 M	4.3×10^{11}
	NaCl + As(III) 3 mM	0 M (LB25 control)	2.7×10^{11}
		0.5 M	1.1×10^{11}
		2 M	1.3×10^{11}
		3 M	1.9×10^{9}

Table 1: Characterization of *B. linens* AE038-8 regarding different growth conditions. Growth at different temperatures is reported as + (growth) and - (absence of growth). Growth of the strain in the presence of heavy metals was semi-quantitatively determined as + (poor growth), ++ (normal growth), +++ (abundant growth) and – (absence of growth), according to the method described by Polti et al. (2007). Growth of *B. linens* AE038-8 in culture medium amended with increasing concentrations of NaCl and NaCl plus arsenite after 48 h of incubation, was quantitatively determined as $CFU.ml^{-1}$. All growth cultures were carried out in LB_{25} broth medium.

A	HSV-1 Field strain Cytopathic Effect Score				
Supernatants from *B. linens*	Serial dilutions				
	1/2	1/4	1/8	1/16	1/32
LB (log)	1	1	1	3	3
	1	1	1	3	3
LB (stationary)	1	1	1	2	3
	1	1	1	2	3
LB + As(III) 3 Mm (log)	1	1	1	2	3
	1	1	1	2	3
LB + As(III) 3 Mm (stationary)	1	1	1	2	3
	1	1	1	2	3
LB + NaCl 2 M (log)	1	1	2	3	3
	1	1	2	3	3
LB + NaCl 2 M (stationary)	1	1	1	2	3
	1	1	1	2	3
LB+ As(III) 3 Mm + NaCl 2 M (log)	1	1	1	2	3
	1	1	1	2	3
LB+ As(III) 3 Mm + NaCl 2 M (stationary)	1	1	1	2	3
	1	1	1	2	3

B	HSV-1 KOS strain Cytopathic Effect Score				
Supernatants from *B. linens*	Serial dilutions				
	1/2	1/4	1/8	1/16	1/32
LB (log)	1	4	4	4	4
	1	4	4	4	4
LB (stationary)	1	4	4	4	4
	1	4	4	4	4
LB + As(III) 3 Mm (log)	1	4	4	4	4
	1	4	4	4	4
LB + As(III) 3 Mm (stationary)	1	4	4	4	4
	2	4	4	4	4
LB + NaCl 2 M (log)	2	4	4	4	4
	2	4	4	4	4
LB + NaCl 2 M (stationary)	2	4	4	4	4
	1	4	4	4	4
LB+ As(III) 3 Mm + NaCl 2 M (log)	2	4	4	4	4
	1	4	4	4	4
LB+ As(III) 3 Mm + NaCl 2 M (stationary)	2	4	4	4	4
	2	4	4	4	4

Table 2 -. Antiviral screening of supernatants from *Brevibacterium* growth. Vero cells grown in 96-well plates were infected with a m.o.i = 0.07 of HSV-1, and treated or not with different supernatants. After 24 h of incubation at 37°C, cytopathic effect from the virus was evaluated and scored with respect to untreated infected control.

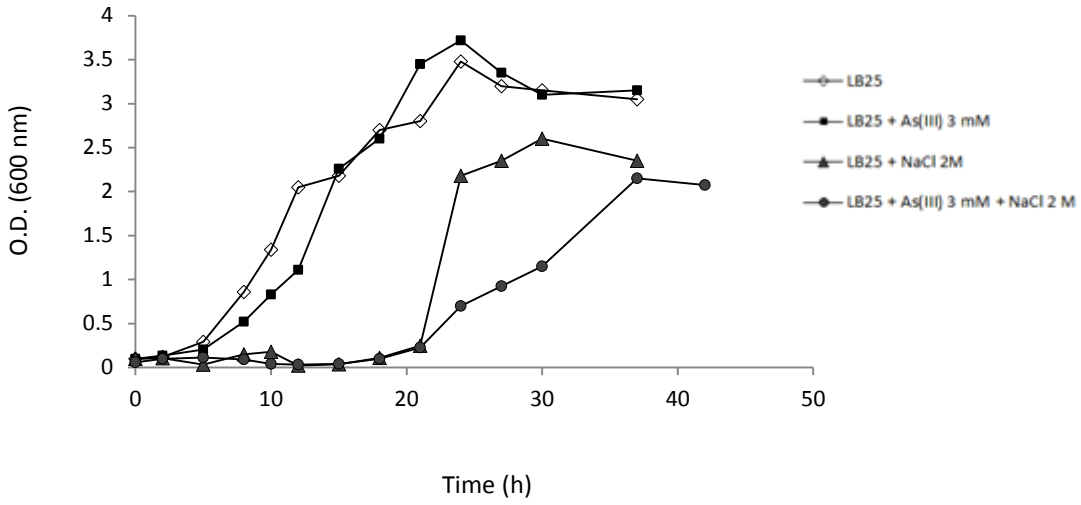

Fig. 1: Growth of *B. linens* AE038-8 in LB_{25} medium plus salts. Empty diamonds: growth in LB25 medium (control). Filled squares: growth in culture medium plus As(III) 3 mM. Filled triangles: growth in culture medium plus 2M NaCl. Filled circles: growth in culture medium plus 2M NaCl and As(III) 3 mM combined.

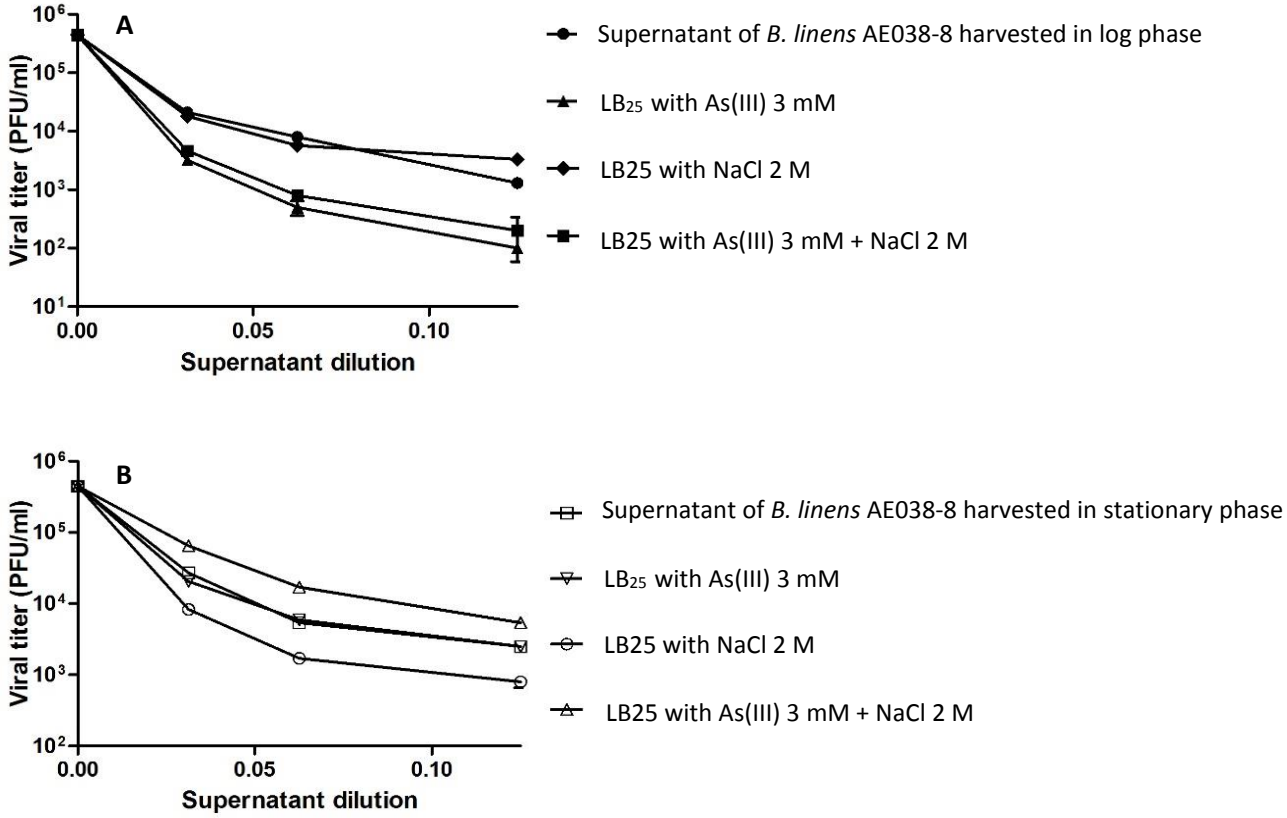

Fig 2 -. Antiviral activity of supernatants. Vero cells grown in 24-well plates were infected with viral yields obtained after infection with HSV-1 Field strain. After 72 h of incubation at 37ºC, cells were fixed, stained with Crystal Violet, and plaque forming units (PFU) were counted. **A:** Supernatants harvested from bacteria in logarithmic phase grown in LB_{25} medium; LB_{25} with As(III) 3 mM ; LB_{25} with NaCl 2 M; or LB_{25} with As(III) 3 mM + NaCl 2 M. **B:** Supernatants harvested from bacteria in stationary phase grown in LB_{25} medium; LB_{25} with As(III) 3 mM; LB_{25} with NaCl 2 M; or LB_{25} with As(III) 3 mM + NaCl 2 M.

Manuscript CMIC-D-19-00508R1

Response to reviewers

Dear Dr. Stackebrandt,

Thank you for giving us the opportunity to submit a revised draft of the "Study of the extremely-tolerant *Brevibacterium linens* AE038-8 with antiviral activity against Herpes simplex virus type 1" for publication in the Journal of Current Microbiology. We appreciate the time and effort that you and the reviewers dedicated to providing feedback on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper. We have incorporated the suggestions made by the reviewers. Please see below, in blue, for a point-by-point response to the reviewers' comments and concerns.

With best regards,

Dr. Laura Alche

Reviewers' Comments to the Authors: Reviewer 1

This manuscript reports the characterization of the resistance of Brevibacterium linens AE038-8, an arsenic hyper-tolerant bacterial strain, to stress factors such as high salt concentrations, heavy metals and high temperature. The ability of a bioactive principle released by this strain to inhibit herpes simplex type 1 multiplication in cell cultures is also described. This is a concise and interesting study, the experimental approach is adequate and results are conclusive and properly discussed. Though the nature of the antiviral principle remains unknown, the pronounced inhibitory action of bacterial culture supernatants against herpes simplex virus is a remarkable contribution to the discovery of new sources of antiviral agents. In reference to figures and tables of the manuscript, I think that some modifications in table 4 and figure 2 would make data presentation clearer.

Specific comments

1- B. linens AE038-8 supernatants exert a higher level of CPE inhibition in HSV-1 Field strain infected cells than in HSV-1 KOS infected cultures. What could be the possible explanation to these results? Did other bacterial products exhibit differential efficacy against HSV strains?

Author response: We cannot give an explanation for the higher level of CPE inhibition observed with the supernatants on HSV-1 Field infected cells with respect to HSV-1 KOS infected ones due to the lack of bibliography on this subject related to bacteria. In fact, publications dealing with this issue refer to antivirals of natural origin (plant, algae, fungus-derived, but not bacteria) effective against HSV-1 and HSV-2, but not against TK- strains of HSV (Current Antivirals and Novel Botanical Molecules Interfering With Herpes Simplex Virus Infection, Front Microbiol. 2020; 11: 139).

2- Table 4

In the first column, it would be clearer if instead of a number, the bacteria growing condition is indicated. For instance, instead of 1 it should say LB (Log), instead of 3 it should say LB- NaCl 2 M (Log), and so on. This change would facilitate the interpretation of the results.

Author response: We agree that the new display of the data looks much more clear and easier to interpret. Changes have been made according to the reviewer suggestion. Note: Because of the re-structuration of tables suggested by reviewer #2 as you will note in the comments below, this table is now re-named as Table 2A and Table 2B. Changes have been highlighted in the blinded manuscript under Track Changes.

3- Figure 2

In line with the previous comment, I suggest replacing the numbers by the bacteria growing conditions in the graphics shown in the figure.

Author response: Changes have been made according to the reviewer's suggestion, and numbers were replaced by growing conditions in the Figure.

4- Legend table 4
Please change "cytopathic effect from the virus was evaluated. CV: Viral Control without treatment." To "cytopathic effect from the virus was evaluated and scored respect to untreated infected control."

Author response: Changes have been made accordingly, and highlighted in the table's text (Please note that this is now Table 2, as mentioned above).

Reviewers' Comments to the Authors: Reviewer 2

This work is based on the study of a bacterial strain of Brevibacterium linens AE038-8 hyper tolerant to arsenic, which was previously isolated from well water in Tucumán, Argentina. The authors focused on characterizing this strain with respect to its resistance to different stress factors, such as salinity and the presence of heavy metals, and evaluated its antiviral capacity against herpes simplex virus type 1 (HSV-1). The experiments carried out are in accordance with the proposed objectives. The study generates novel contributions in the area of knowledge of antiviral agents, particularly against the HSV-1 virus. Based on the aforementioned, I consider that the manuscript should be accepted for publication in this journal.

Author response: We appreciate your comment and consideration, as well as your positive feedback.

On the other hand, given the limitations of space that the authors have due to the number of pages that are stipulated by this journal, and understanding that it would not be convenient to remove the information presented, I suggest that the results reported in Tables 1, 2 and 3 could be condensed into a single table.

Author response: We have condensed tables 1, 2 and 3 into a single one, which is now renamed as Table 1.

Note: table's numbers have been changed accordingly and highlighted in the blinded manuscript under track changes.

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