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Occurrence of Resistance to Antibiotics, UV-B, and Arsenic in Bacteria Isolated from Extreme Environments in High-Altitude (Above 4400 m) Andean Wetlands

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Abstract High-altitude Andean wetlands are pristine environments with extreme conditions such as high UV radiation, high heavy metal content (mainly arsenic), high salinity, and oligotrophy. In this paper, the UV-B resistance and tolerance to arsenic of phylogenetically characterized bacteria (Actinobacteria [six isolates], Firmicutes [four isolates], and γ-Proteobacteria [three isolates]) isolated from Laguna Vilama (4400-m altitude) and Laguna Azul (4560 m) were determined. In addition, given that multiple antibiotic resistances were also determined, a relationship between antibiotic resistances as a consequence of mutagenic ability or in relation to metal resistance is proposed. High UV-B resistances were found, since after 30 min (0.7 KJ m^{-2}) and 60 min (1.4 KJ m^{-2}) of irradiation, most of the studied bacteria did not show a decreased survival; what is more, many of them had an improved survival with the increased doses. Augmentations in mutagenesis rates were observed after UV-B irradiation in only 4 of the 13 tested isolates. Arsenite tolerance was also established in 8 of the 13 tested strains: *Staphylococcus saprophyticus* A3 and *Micrococcus* sp. A7, which were able to grow in media containing up to 10 mM As(III). Finally, predominance of antibiotic resistances (azithromycin, erythromycin, clarithromycin, roxithromycin, streptomycin, chloramphenicol, gentamycin, kanamycin, tetracycline, and ampicillin) was found, in all the isolated strains from both wetlands, with unexpectedly high minimal inhibitory concentrations (MICs; >2 mg mL^{-1}) for macrolides. These results demonstrate that in extreme environments like high-altitude wetlands there is a correlation of multiresistances to UV-B radiation and arsenic, and that antibiotic resistances are also widespread in these pristine environments, where antibiotic selective pressure is supposed to be absent.

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Introduction

Extreme environments are interesting as sources of microorganisms with exceptional phenotypic and genotypic characteristics. Bacteria that inhabit these environments need to develop efficient mechanisms and strategies to thrive under such conditions. Exposure to UV radiation is considered to be especially harmful to microorganisms because they have haploid genomes with little or no functional redundancy, because they are small, and because they lack thick, protective cell walls [9, 13, 17, 23, 24]. Under extreme UV stress, bacteria are known to increase mutational events, as a last resistance mechanism called error-prone repair [18, 20, 22]. This system enables lesion bypass during DNA replication. Under normal conditions, such lesions would block replication by DNA polymerase III. But in the presence of UV-radiation damage, this bypass allows the cell to replicate its DNA and survive in the presence of unrepaired lesions. For obvious reasons, this results in high numbers of mutations [22]. In many cases, spontaneous resistance to antibiotics (ATBs) is known to emerge under such mutagenic conditions, as a consequence of mutagenesis of modified potential target genes; for example, rifampicin (Rif) resistance is

commonly due to mutations in the β subunit of RNA polymerase, encoded by the *rpoB* gene. In fact, bacterial mutagenic capacity after exposure to UV stress is measured by an increase in resistance to Rif and/or nalidixic acid [10, 13]. Therefore, our hypothesis was that it would be possible to find ATB-resistant bacteria in extremely UV-irradiated environments.

On the other hand, there is growing concern that the presence of heavy metals functions as a selective agent in the proliferation of resistance to antibiotics. Documented associations between the types and levels of metal contamination and specific patterns of antibiotic resistance suggest that several mechanisms underlie this coselection process [2, 3, 7, 12, 14, 15]. These coselection mechanisms include coresistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals). Indirect but shared regulatory responses to metal and antibiotic exposure such as biofilm induction also represent potential coselection mechanisms used by prokaryotes. Metal presence, therefore, represents a long-standing, widespread, and recalcitrant selection pressure with both environmental and clinical importance that potentially contributes to the maintenance and spread of antibiotic resistance factors [2]. This correlation has been largely established in contaminated environments [12],but it has never been tested in pristine environments, where artificial inputs of antibiotics do not occur. Andean wetlands are of special interest as extreme environments to study ATB resistance associated with UV irradiation and/or heavy metal resistance, since these locations share a group of interesting characteristics. (i) They are pristine and isolated with no access roads. (ii) They are distant from each other (more than 500–700 km). (iii) They are located at high altitudes (4200 to 4660 m above sea level [asl]) and surrounded by desert, implying that few clouds shade the UV irradiation. (iv) They are oligotrophic, resulting in deep UV penetration in the water column. (v) They are exposed to extreme conditions, such as high salinity and high arsenic content (of geochemical origin), among others.

In previous papers, UV-B-resistant bacteria have been isolated by direct irradiation of Laguna Azul water. Molecular identification of these isolates resulted in a predominance of Gram-positive bacteria [6]. Additionally we compared *Acinetobacter johnsonii* A2 with environmental strains isolated from less exposed environments. We found that in this case UV-B resistance was consistent with the irradiation levels in their native environment [7].

In the present paper we isolated bacteria from Laguna Vilama alone and together with isolates from Laguna Azul. We determined their resistance to ATB, arsenic, and UV, in relation to their capacity for mutagenesis.

Materials and Methods

Description of Bacterium Isolation Environments

Both lakes are located in the northwest of Argentina. *Laguna Azul* is an oligotrophic lake located at 4560 m asl. It is part of the Salar de la Laguna Verde in the Andean region of Catamarca province, Argentina (27°34′S, 658°32′W). The location is a very isolated site, with no access roads. Rainfall is scarce so the lakes are shallow and present a high metal content [20]. Measured arsenic content was 0.014 mg/L, and salinity was 0.112 mg/L. In the sampling day at noon, in the austral summer, the maximal UV-B irradiance reached 10.8 Wm^{-2} for the 300 to 325 nm range.

Laguna Vilama is located in the plateau of Jujuy Province at 4600 m asl, (22°30'S, 66°50'W). Climatic and geographical conditions are similar to those at Laguna Azul. The arsenic concentration found in this wetland was 3.1 mg/L, and the salinity 90, during the dry season. It is included in the List of Wetlands of International Importance (RAMSAR).

Sampling and Strain Isolation, Cultivation, and Identification

Samples were collected during the Austral summer in Laguna Azul and Laguna Vilama. Surface water samples were collected in sterile flasks and stored at between 5 and 10°C until further processing in the laboratory, within approximately 24 h.

Some strains from Laguna Azul (identified by an A in their strain code) have been described previously [6]. Strains isolated from Laguna Vilama (V in their strain code) were identified in the present study (Table 1). Bacteria were isolated by plating in wetland water enriched with Luria Bertani (LB) 0.1× medium plus agar. Cultivation in other enriched media showed no differences in the number and diversity of cultivable bacteria (data not shown). Colonies were grouped into morphologically different types, and homogeneity of different colony groups was corroborated by Rep polymerase chain reaction (data not shown). Isolated strains were cultured in LB broth, with shaking, at 20°C. Genomic DNA extraction, 16S rDNA gene partial amplification, and sequencing were carried out as described by Fernández Zenoff et al. [6]and resulting sequences registered in Genbank (Table 1).

UV-B Irradiation

In order to quantify the effect of UV-B on the strains, cells in mid log phase of growth were harvested by centrifugation (7000 rpm [Eppendorff] for 15 min at 4°C) and

Table 1 Strains isolated from Laguna Vilama and Laguna Azul

Closest related species	Accession no.	% 16S rDNA similarity	Resistance													
			D_{37}	D_{90}	Mutagenesis induction ratio (MF_T/MF_{UT})	As(III)	Em	Clm	Azt	Rox	Sm	Km	Gen	Amp	Ams	Tc	Cm
Firmicutes																	
Bacillus pumillus A4	DQ217665	99	$3.0^{1.2}$	$1.4^{0.4}$	1.83	+	+	+	+	+	+	−	−	+	−	−	−
Bacillus vallismortis V8	AM235883	100	$3.4^{0.02}$	$1.4^{0.0}$	$2.44^{(0.4)}$	−	+	+	+	+	+	+	−	+	+	+	+
Bacillus vallismortis V3	AM235882	100	$2.7^{1.7}$	$1.1^{0.0}$	—	−	+	+	+	+	−	−	−	+	−	+	+
Staphylococcus saprophyticus A3	DQ112023	97	$2.9^{0.03}$	$1.6^{0.0}$	—	+	+	+	+	+	+	−	+	+	−	−	−
HGC																	
Micrococcus sp. A1	AM403127	98	$1.4^{0.00}$	0.0	0.97	+	+	+	+	+	+	+	+	+	−	−	+
Micrococcus sp.V7	AM403126	98	$3.0^{1.07}$	$1.5^{0.5}$	—	+	+	+	+	+	+	+	−	+	−	+	+
Nocardia sp. A5	DQ112024	99	$8.3^{7.7}$	$2.6^{1.3}$	—	−	+	+	+	+	−	−	−	+	−	−	−
Micrococcus sp. A7	AM235879	99	$10^{0.00}$	$2.6^{0.0}$	—	+	+	+	+	+	−	−	−	+	−	+	+
Rhodococcus erithropolis V2	AM236137	97	$3.3^{0.02}$	$1.6^{0.1}$	1.46	+	+	+	+	+	+	−	−	+	+	−	−
Brachybacterium sp. V5	AM236138	97	$5.4^{0.00}$	$1.7^{0.0}$	—	+	+	+	+	+	−	+	−	+	+	−	−
γ-Protobacteria																	
Pseudomonas sp. V1	AM403128	98	$9.0^{6.67}$	$1.2^{1.1}$	$3.37^{(3.3)}$	−	+	+	+	+	+	−	−	+	+	−	+
Enterobacter sp. V4	AM403125	98	$3.0^{0.02}$	$1.4^{0.1}$	0.89	−	+	+	+	+	+	+	−	+	+	+	+
Acinetobacter johnsonii A2	AY963294	99	$16.8^{17.2}$	$2.3^{0.4}$	4.36	+	+	+	+	+	+	+	−	+	−	−	−

resuspended in 0.9% saline solution. One hundred microliters of appropriate serial dilutions (10^{-3} to 10^{-6}) were spread onto LB-agar plates (9 cm in diameter) in duplicate and then covered with an acetate film to block out UV-C radiation. Cells were irradiated at 15°C with UV-B lamps (09815-06; Cole Parmer Instruments Co.), with major emission at 312 nm. Irradiance was quantified with a radiometer (09811-56; Cole Parmer Instruments Co.) for 312 nm with a half-bandwidth: of 300 to 325 nm. The radiation intensity measured under the plates was 0.38 W m^{-2}. After 30 min (0.7 KJ m^{-2}) and 60 min (1.4 KJ m^{-2}) of exposure, the plates were removed and the number of colony-forming units (CFUs) was determined after 24 h of incubation at 25°C in the dark to prevent photoreactivation. Controls of unexposed samples were run simultaneously in darkness and calculations of the percentages of cell survival after each treatment were made relative to these controls. Results are expressed as D_{37} and D_{90}, which indicate the radiation required to inactivate 63% and 10% of a bacterial population, respectively [2]. The D values were calculated from the regression line of the exponential decrease of CFU with time. The r^2 of the regression is provided as indication of the goodness of fit.

UV-B-Induced Antibiotic Resistance (Mutagenesis)

Bacteria exposed to UV-B and control bacteria were plated onto duplicate plates containing 20 μg/ml Rif to determine the percentages of Rif-resistant mutants under both treatments. Plates were incubated at 25°C for 16 h and viable Rif^+ mutants (CFU) were counted. Following incubation, the mutation percentages were determined comparing Rif resistances in unexposed controls. Bacterial strains were streaked onto plates with Rif (20 μg/ml) and incubated at 25°C for 24 h; growth was compared to that on plates without Rif added. The mutagenic increased was measured by dividing the mutant frequency of nonirradiated (untreated) cells (MFut) by the mutants frequency of irradiated cells (MFt).

Antimicrobial Susceptibility

Isolates were tested by disk diffusion methods on Müeler-Hinton agar according to the current recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) for susceptibility. ATBs tested were as follows: Azt (azithromicyn), 50 μg; Em (erythromycin), 50 μg; Clm (clarithromycin), 50 μg; Rox (roxithromycin), 50 μg; Sm (streptomycin), 20 μg; Cm (chloramphenicol), 30 μg; Gen (gentamycin), 10 μg; Km (kanamycin), 20 μg; Tc (tetracycline), 20 μg; and Amp (ampicillin), 100 μg. Since there are no standard values for MICs (minimal inhibitory concentrations) in environmental strains, susceptibility to increasing ATB concentrations was determined. The chosen ATB was Azt because of its epidemiological relevance as it is a third-generation macrolide derived from Em. Fresh cultures (0.5 OD) were inoculated in LB broth containing Azt at different concentrations (500 to 2500 μg ml^{-1}). After 24 h of incubation, cultures were plated at several dilutions in LB agar plates containing serial dilutions of the test ABT. Plates were incubated at 30°C for 24 h, and the bacterial colonies were then counted. Tests were done in duplicate.

Arsenic Resistance Test

Overnight cultures of all strains were inoculated in new fresh LB broth in duplicate containing sodium arsenite (As[III]) at final concentrations of 2.5, 5, and 10 mM. After incubation at 30°C during 24 h, 100 μl of the appropriate serial dilution in 0.9% NaCl was spread on LB plates with 2.5, 5, and 10 mM sodium arsenite, respectively. The number of CFU was determinate after 24 h of incubation at 30°C. Strains that did not grow at 2.5 mM arsenite were considered to have no resistance to the bacteria.

Results

Identity of Isolates

The numbers of culturable bacteria were 3×10^3 and 2.5×10^3 per mL in Laguna Azul and Laguna Vilama, respectively. Affiliations of isolates A4, A3, A5, and A2 from Laguna Azul have been determined in a previous publication. The strains phylogenetic diversity was evaluated by partially sequencing the 16S rRNA genes of the seven isolates from Laguna Vilama and three from Laguna Azul. In sum, sequences belonging to three bacterial phyla—Actinobacteria (six isolates), Firmicutes (four isolates), and γ-Proteobacteria (three isolates)—were found. Actinobacteria were represented by *Micrococcus* sp. A1, A7, and V7, *Rhodoccoccus* sp. V2, *Nocardia* sp. A5, and *Braquibacterium* sp.V5. *Firmicutes* were represented by *Bacillus vallismortis* V8 and V3, *Bacillus pumillus* A4, and *Staphylococcus* sp. A3. γ-Proteobacteria were represented by *Acinetobacter johnsonii* A2, *Pseudomonas* sp. V1, and *Enterobacter* sp. V4 (Table 1).

UV-B Resistance and Mutagenesis Ability

The corresponding D_{90} and D_{37} values after artificial UV-B exposure are shown in Table 1 (D_{90} and D_{37} are the radiation doses required to inactivate 10% and 63%

of the bacterial population, respectively). All the isolates showed some resistance to UV-B since none of them decreased their survival below 25% of the initial cell concentration.

In most cases there were no significant differences between the two irradiation doses assayed (Fig. 1). Three isolates (V7, A3, and V2) did show further inhibition with higher doses of UV radiation. Finally, isolate V3 showed increased survival at the higher dose.

UV-B-induced mutations were found in only 4 of the 13 tested strains: A4, V8, V2, and A2 increased the Rif resistance 2.7, 2.2, 1.2, and 3.9 times, respectively, after irradiation treatment. The other strains presented no UV-induced mutagenesis. As strain *Acinetobacter johnsonii* A2 presented the highest UV-B-induced mutations, we measured UV-B-induced mutations in a reference strain of the same species, *Acinetobacter johnsonii* ATCC 17909, as an external control to strain A2. Our results showed that after UV irradiation no increase in UV mutagenesis was found in the reference strain, suggesting that the mutagenic ability of strain A2 could be related to the conditions in the isolation environment.

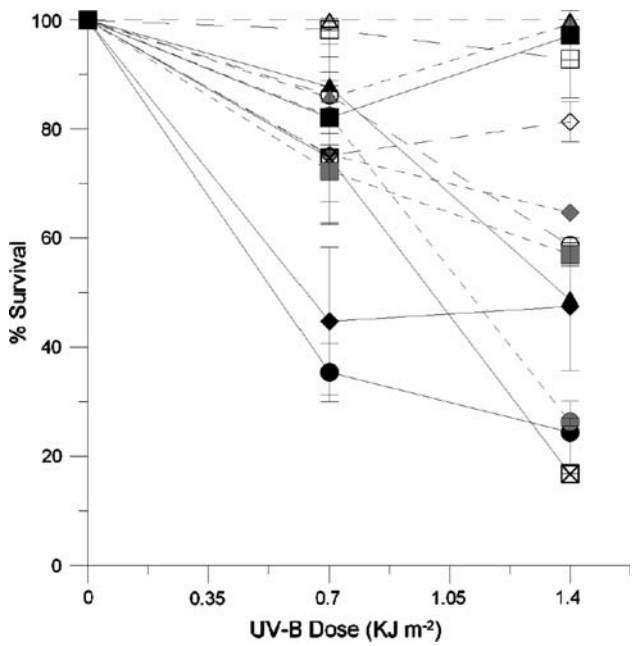

Fig. 1 UV-B resistance percentage from Laguna Vilama and Laguna Azul strains (called V and A, respectively) at different exposition doses: —♦—, *Micrococcus* sp. A1; —■—, *Acinetobacter johnsonii* A2; —▲—, *Staphylococcus saprophyticus* A3; —•—, *Bacillus pumillus* A4; –◇–, *Nocardia* sp. A5; –□–, *Micrococcus* sp. A7; –Δ–, *Pseudomonas* sp. V1; –○–, *Rhodococcus erithropolis* V2; ⊠, *Bacillus vallismortis* V3; ···■···, *Enterobacter* sp. V4; ···◇···, *Braquibacterium* sp. V5; ···○···, *Micrococcus* sp. V7; ···Δ···, *Bacillus vallismortis* V8

Antibiotic Resistance Profile

Considering the pristine nature of the original environment high levels of resistance to ATB were found in the isolates. All the isolates were resistant to at least 8 of the 11 tested ATBs (Table 1). Moreover, all of them were resistant to Amp and to all the MCLs tested (Em, Clm, Rox, and Azt).

MIC is defined as the lowest concentration of drug that inhibits more than 99% of the bacterial population. The MIC is well established for clinical isolates. However, no information is available in the NCCLS about environmental isolates like Actinobacteria and Bacilli (with the sole exception of *Bacillus anthracis*, with a MIC for MLC of 0 to 8 μg ml^{-1}). In order to clarify whether the MICs found in extreme environment isolates were similar to those found in clinical isolates, we determined MICs for MLC (Azt). As shown in Fig. 2, in the environmental isolates MICs were elevated (ranging from 0.5 to 2 mg mL^{-1}) compared with those in studies performed with Gram-positive and Gram-negative clinical isolates (0.05 to 0.1 mg mL^{-1} for Azt [18, 20]). What is more, inhibition was not achieved in strains A2 and A1 at the highest tested concentration. In addition, strain A2 seemed to increase its survival with Azt increasing concentrations (Fig. 2).

Arsenic Resistance

Arsenite (As[III]) resistance was determined. We used arsenite instead of arsenate (As[V]) because the first is more toxic to the cell. As(III) resistance was also found in 8 of the 13 tested isolates that survived at 5 mM As(III), but at 10 mM growth was only found in strains A2, V2, V7, A3, and V5 (Fig. 2).

Discussion

Pristine high-altitude wetlands in Andean mountains provide an opportunity to obtain microbes from a planktonic environment that have experienced long-term exposure to high UV radiation, cold temperatures, high arsenic concentrations, salinity, and low carbon availability. Strains representative of the bacterioplankton community should carry traits that have allowed them to adapt to these conditions. Phylogenetic diversity found in cultivable isolates of Laguna Vilama denoted a predominance of Gram-positive bacteria; these results are in accordance with our previous study in Laguna Azul [23]. Predominance of Gram-positive bacteria in high-altitude wetlands could be related to UV-radiation stress. The Gram-positive cell wall characteristics may protect them from radiation. In addition, in some Gram positives other characteristics may help resistance, such as the spore forming ability in *Bacillus* and

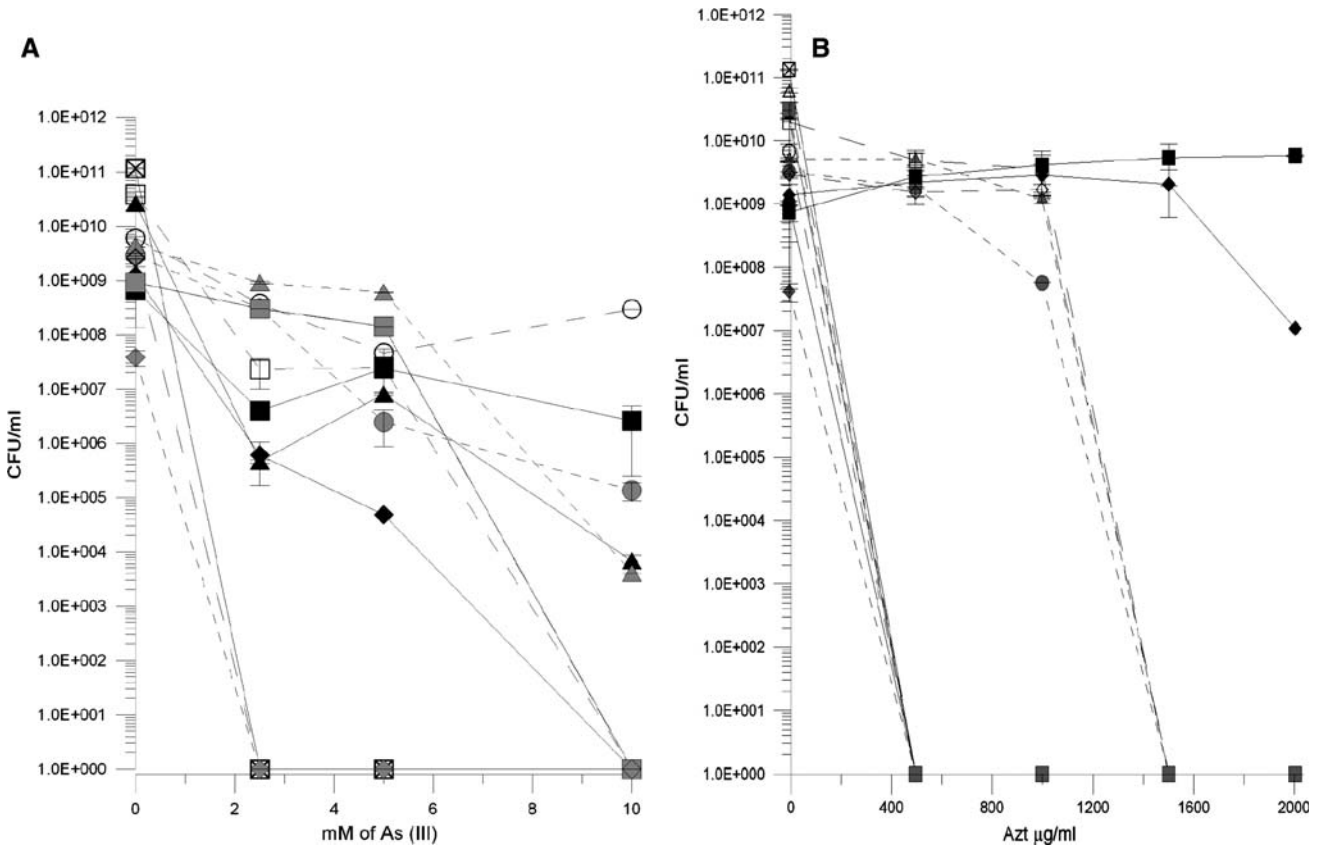

Fig. 2 (**A**) Colony-forming units (CFU) per milliliter at different sodium arsenite concentrations. (**B**) CFU at different azithromycin concentrations: —♦—, *Micrococcus* sp. A1; —■—, *Acinetobacter johnsonii* A2; —▲—, *Staphylococcus saprophyticus* A3; —•—, *Bacillus pumillus* A4; –◇–, *Nocardia* sp. A5; –□–, *Micrococcus* sp. A7; –Δ–, *Pseudomonas* sp. V1;–○–, *Rhodococcus erithropolis* V2; ⊠, *Bacillus vallismortis* V3; ··· ■···, *Enterobacter* sp. V4; ···◇···, *Braquibacterium* sp. V5; ···○···, *Micrococcus* sp. V7; ···Δ···, *Bacillus vallismortis* V8

the relatively high GC content in Actinobacteria. Previous reports analyzing the total bacterial community in alpine lakes have reported a predominance of Actinobacteria and have proposed that their abundance may be related to their low AT content and the consequent UV resistance [22]. On the other hand, Agogue et al. found no correlation between the resistance of bacterial species and their GC content in marine isolates [1]. Thus our speculations should be sustained in further analyses of total community composition. Very few microbiological studies have determined the microbial resistance to the diverse extreme conditions in these environments: Demergasso et al. [5] analyzed the distribution of prokaryotic genetic diversity in relation to salinity in athalassohaline lakes of the Atacama Desert, northern Chile, and observed that the relative abundance of the Bacteroidetes group increased with salinity content. Predominance of β-proteobacteria has been reported from Laguna Pozuelos in the Argentinean Puna (3600-m altitude) [6], and predominance of Gram-positive bacteria with high UV resistance was also reported from Laguna Azul (4520-m altitude) and Laguna Pozuelos [6, 7]. But to our knowledge no studies have been carried out integrating and/or comparing resistance to different factors in these environments. We determined three kinds of resistances in our isolates: UV, arsenic, and ATBs. We selected these stress factors because both UV and As content are widespread extreme conditions in these wetlands. ATB resistance was also studied in order to determine if UV resistance and its effect on mutagenic ability were correlated with ATB resistance in a pristine environment. Additionally, we wanted to explore whether there was also an association between ATB and heavy metal resistance, since this association had been only established in contaminated environments [7]. Our results showed that the analyzed resistances were largely widespread in most of the isolates from both environments.

We expected to find UV-B-resistant bacteria in bacterial communities from high UV-irradiated environments. D37 and D90 values correlated well with UV-B resistance in all bacteria. In that way the most resistant bacteria—*Acinetobacteri johnsonii* A2, *Nocardia* sp. A5, *Micrococcus* sp. A7, and *Pseudomonas* sp. A1—showed high D37 and D90 values. However, despite the high UV resistances found, we observed that UV-induced mutagenesis was not a

widely distributed phenomenon, indicating that error-prone repair could not be a common strategy to survive UV stress. An alternative explanation could be that the doses used in these experiments are too low to trigger the error-prone repair system, and then lesions would not be repaired by mutagenesis mechanisms. This is the case in *E. coli* and many other bacteria, where expression of dark repair mechanisms is differentially inducible, depending on the amount of DNA damage experienced by the cells [22, 26]. More experiments with increased UV-B doses should be done to study this possibility.

The presence of widespread resistance to ATB was more unexpected in these pristine and oligotrophic environments, where it is not predictable to find selection pressure for ATB resistance, particularly to synthetic ATBs like Azt, Rox, and Clm. The presence of ATBs in the water is ruled out since no antimicrobial component production against Gram-positive and Gram-negative bacteria was detected in the lake water. What is more, a similar ATB resistance pattern was found in the two lakes, which are geographically separated but share similar environmental conditions (oligotrophy, UV incidence, As content, etc.). We also found that the wide ATB resistances do not correlate well with UV-B-induced mutagenic ability. Therefore our results demonstrate that, in our isolates, the presence of ATB resistance would not be induced by mutagenesis. In contrast, as ATB and As resistances were found in most of the isolated strains, we assume that there could be a codistribution of ATB and metal resistances. To our knowledge this coresistance is established for the first time in bacteria isolated from pristine environments. Further studies establishing the molecular determinants of these resistances should be performed in order to test these hypotheses. On the other hand, it was established that both heavy metals and UV radiation cause oxidative stress in bacteria.

Finally, we would like to emphasize the high resistance of *Acinetobacter johnsonii* A2 to the tested stresses, since its survival ability was enhanced with increased UV, As, and ATBs. The high UV-B-induced mutagenesis presented by this "super" strain could be associated with a resistant phenotype. Results found in the reference strain ATCC17909 would support this idea.

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