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Diverse UV-B Resistance of Culturable Bacterial Community from High-Altitude Wetland Water

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Abstract. Isolation of most ultraviolet B (UV-B)-resistant culturable bacteria that occur in the habitat of Laguna Azul, a high-altitude wetland [4554 m above sea level (asl)] from the Northwestern Argentinean Andes, was carried out by culture-based methods. Water from this environment was exposed to UV-B radiation under laboratory conditions during 36 h, at an irradiance of 4.94 W/m^2. It was found that the total number of bacteria in water samples decreased; however, most of the community survived long-term irradiation (312 nm) (53.3 kJ/m^2). The percentage of bacteria belonging to dominant species did not vary significantly, depending on the number of UV irradiation doses. The most resistant microbes in the culturable community were Gram-positive pigmented species (*Bacillus megaterium* [endospores and/or vegetative cells], *Staphylococcus saprophyticus*, and *Nocardia* sp.). Only one Gram-negative bacterium could be cultivated (*Acinetobacter johnsonii*). *Nocardia* sp. that survived doses of 3201 kJ/m^2 were the most resistant bacteria to UV-B treatment. This study is the first report on UV-B resistance of a microbial community isolated from high-altitude extreme environments, and proposes a method for direct isolation of UV-B-resistant bacteria from extreme irradiated environments.

Bacteria may account for up to 90% of the cellular DNA in aquatic environments. These organisms play a central role in the cycling of nutrients in aquatic ecosystems and they constitute a fundamental link in the carbon transfer process (i.e., the microbial loop [3]). Therefore, the study of factors that control bacterial growth in the environment is of primary importance. Among the different factors that affect bacterial growth, solar ultraviolet radiation (UV-B, 280-310) has only recently received attention. UV-B could be particularly deleterious for bacteria because these organisms have simple haploid genomes with little or no functional redundancy and they are small, which precludes effective cellular shading or protective pigmentation [10]. On the other hand, the percentage of cells of bacteria belonging to dominant species should vary significantly depending on the intensity of UV irradiation. In that way, the UV irradiation of plankton samples would reveal less abundant species, which are primarily UVR-resistant bacteria. Thus, UV irradiation can be used as a selective factor for the isolation of UV-resistant species.

Limited knowledge on molecular and physiological responses to UVR is available for environmentally relevant bacteria, less still in extreme UV-B environmental isolates. Studies of photobiological responses of bacteria that live in saline aquatic and hypersaline terrestrial environments have been carried out [7, 9, 11, 13, 15], but we are not aware of any performed under extremely high UVR regimes as those found in the Andean wetlands in the subtropics (4560 m above sea level [asl]).

In this article, we report the diverse response, by measuring survival, to long-term UV-B exposure of a culturable community of bacteria that inhabits a high-altitude wetland such as the Laguna Azul, a saline lake located in the Argentinean northwestern Andes at 4560 m asl.

Materials and Methods

Study area. 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

*This article is dedicated to the memory of Carolina Colin.
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Table 1. Phylogenetic affiliation of selected isolated strains

Strain	Phenotype	GenBank access N°	Closest related	% 16S rDNA similarity	Microbial classification
A1	Yellow	DQ112025	*Bacillus megaterium*	96%	Gram-positive LGC content
A2	White	AY963294	*Acinetobacter johnsonii*	99%	γ-Proteobacteria
A3	Soft orange	DQ112023	Staphylococcus saprophyticus	97%	Gram-positive
A4	White	DQ217665	*Bacillus pumillus*	99%	Gram-positive LGC content
A5	Strong orange	DQ112024	*Nocardia* sp.	99%	Gram-positive HGC content

Catamarca province, Argentina (26° to 28° S; 65° to 67° W). This is an isolated site with no access roads. In this area, some of the highest mountains of the Andean system are located. The water temperature was 5°C at the sampling moment (13:30 h). On the sampling day, the maximal UV-B irradiance reached 10.78 W/m^2 for 312 nm (half bandwidth 300–325 nm).

Sampling. Surface water samples were collected during December 2003 (near the beginning of austral spring) in 10-L acid-washed polyethylene bottles, after prerinsing the containers with lake water. The water was stored at 4°C until further processing in the laboratory (within approximately 24 h after collection), which is located 800 km away from the sampling site.

UV-B irradiation. An aliquot of 250 mL of Laguna Azul water was transferred to 500-mL sterile Plexiglas containers in duplicate. These were covered with acetate film to block out UV-C, and then they were placed in an incubator with a low temperature shaker (C25K, New Brunswick Scientific, Edison, NJ) at 4°C with slow shaking (25 rpm) during 36 h exposed to UV-B (two Lamps 09815-06, Cole Parmer Instruments Company, with major emission line at 312 nm). Irradiance was quantified with a radiometer (09811-56, Cole Parmer Instrument Company) for 312 nm with half bandwidth: 300 to 325 nm. The UV-B irradiance was 4.94 W/m^2. Aliquots were extracted at different times, 100 μL from 30 times concentrated by centrifugation at 13,000 rpm sample, and were plated in duplicate. All isolates were distinguished on agar plates by color, colony surface, and size. Because it is known that the kind and degree of pigmentation also depend on the media used and growing conditions, the homogeneity of each different colony group was corroborated by repetitive extragenic palindromic (Rep) polymerase chain reaction (PCR) (data not shown). The colonies were grouped into five morphologically different types. This procedure allowed us to determine the number and diversity of culturable bacteria present in Laguna Azul along the UV-B irradiation treatment. The lake water was scanned in order to study its ability to absorb terrestrial UV radiation, and no absorption was found.

Bacterial strains and growth procedures. Plating of different sampling times was made in 0.22 μm (Biopore filters) filtered lake water plus 1.2 % (wt/vol) agar (Difco).

All bacteria were grown in LB (Luria Broth) at 25°C with shaking.

Determination of phylogenetic affiliation of the selected bacteria. Genotypic characterization of isolated strains was performed with single colonies. For DNA extraction and 16S rDNA amplification, standard protocols used in Ferrero et al. [4] were used. Universal primers were used to amplify 16S rDNA gene (8-27F and 1492). PCR products were checked in 0.8 % (w/v) agarose gels, and DNA sequencing was performed by Macrogen Inc. (Korea). The sequences were registered in the GenBank Data Library (Table 1).

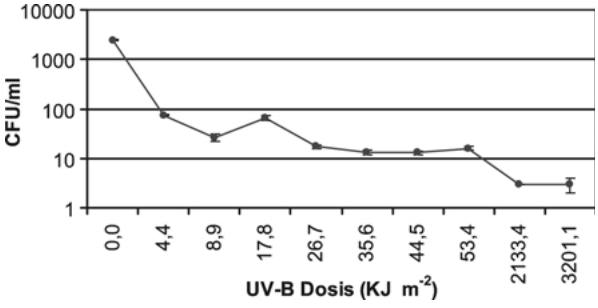

Fig. 1. Survival culturable bacteria along long-term UV-B exposure. Data are the median of two independent experiments (Student t test performed with a $P < 0.05$ found).

Results

Azul lake water plus agar has been implemented as the ''medium of choice'' for culturing and enumerating the bacterial culturable community. Cultivation in other reach mediums showed no differences in the number and diversity of culturable bacteria (data not shown). Because the lake water was not analyzed for spore-forming and non-spore-forming bacteria, we cannot discard the possibility that spore-forming ones were selected with UV-B radiation.

As can be observed in Figure 1, an exponential decrease of almost 3 magnitude orders in the number of cfu of total community was determined at the end of the first hour of UV-B irradiation (17.8 KJ m^{-2}). Between the second and the third hours of exposure, a soft increase of cfu could be determined. In the fourth hour, cfu fell again until the 24th hour, where the cfu reached only one order of magnitude, until the end of the exposure (36 h), represented only by strain A5 (Fig. 2). Statistical analysis was done for the significance between the exposed bacteria strains. The Student t test was performed and $P < 0.05$ was found.

The succession and survival of bacteria from water collected at Laguna Azul after a long-term exposure to UV-B are shown in Figure 2. With the unique exception of strain 4 that did not survive the first 30 min of exposure, all the strains were still resistant during the first 6 h of UV-B exposure (106 KJ m^{-2}) with variability in the dominance of strains comparing time 0. At this

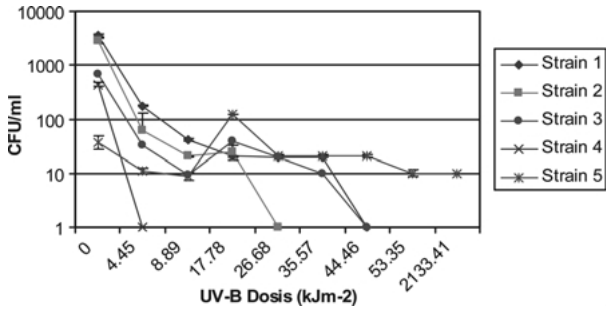

Fig. 2. Survival curves in Laguna Azul community bacterial culturable strains during long-term UV-B exposure. The data are based on two different platings.

sampling time, strain A1 maintained its dominance with a 40% of total community, whereas the orange-colored strain A5 that was almost unrepresented in the beginning of the exposure reached 20% of total community. The same increase happened with strain A3, but in this case, strain A3 was almost unrepresented between 3 and 5 h of exposure (53.3 to 88.9 KJ m^{-2}) and appeared again at 6 h. Strain A2 decreased from 35% in the beginning to 20% at 6 h of exposure (88.9 KJ m^{-2}).

The dominance of strain A5 became absolute in the 24 h of exposure until the end of the experiment at 36 h (640.2 KJ m^{-2}). This strain was almost unrepresented in the community composition at time 0, increased its abundance temporarily in the second hour of exposure, and survived after 36 h of exposure to UV-B (doses). The D37 values of this strain were 30% higher that the other isolated strains. The D_{90} values could not be determined, because strain A5 never achieved the 90% inhibition during the whole exposure experiment (Table 2).

The Laguna Azul isolates A1, A2, A3, A4 and A5 were identified by 16S rDNA sequence analyses as species of the genera *Bacillus megaterium, Acinetobacter johnsonii, Staphylococcus saprophyticus, Bacillus pumillus*, and *Nocardia* sp., respectively, based on the proximities of their sequences to type strains. Their access numbers in GenBank Data Library are DQ112025, AY963294, DQ112023, DQ217665, and DQ112024, respectively (Table 1).

Discussion

In our study location, with these UV-B values, it was rather expected to find UV-B (and UV-A because parts of were applied with UV-B lamps) UV-A resistant bacteria. Bacteria regarded as tolerant or resistant to UVR were recovered from solar radiation–exposed habitats, including aquatic, and halophiles habitats, spacecraft assembly, great salt plains in Oklahoma, and the plant phyllosphere [1, 2, 7, 8, 9, 11, 12, 13, 14, 15]. However, there are authors that found little or no correlation between UVR resistance and the natural levels of solar radiation exposure [5]. It does not seem to be the case in the culturable bacteria from Laguna Azul, where a general UV-B resistance was found in most of the culturable community. Only one of the five strains found did not survive the first UV-B exposure hour, whereas the others demonstrated a general high UV-B resistance that allowed them to survive doses of 106.7 KJ m^{-2} or even more than 640.2 KJ m^{-2} of UV-B (36 h) (Fig. 2).

Concerning the bacterial identification, to our knowledge, this is the first report of UV-B resistance of *Nocardia* sp. and *Staphylococcus saprophyticus*. On the other hand, *Bacillus megaterium* spores were previously found in spacecraft assembly facilities and exhibited resistance to UV-C radiation (254 nm). Interestingly, the spores of this isolated strain exhibited two to three times higher LD90 values in response to UV than did the reference strain *B. subtilis* ATCC 6633 [11].

Most of the isolated were Gram-positive strains, spore-forming or HGC bacteria; therefore, perhaps it is to be expected that Gram-positive bacteria would have a better adaptation to UV stress, just because of their cell wall characteristics, or because of their spore-forming ability in *Bacillus*, or low AT content in *Actinomycetes* (remembering that thymine dimmers are the main target in UV-B damage). Unpublished data from our laboratory in total community molecular determination by DGGE in this lake suggest that this Gram-positive bacteria predominance is also found in whole bacterial community (culturable and not yet culturable).

The pigmentation in bacteria is thought to be insufficient to explain the resistance of these cells to UV-B irradiation, because the small size of the cells probably precludes effective protection by pigmentation [10]. In this case, pigmentation of strains A5, A1, and A3 probably could be a complement to UV-B resistance. Preliminary results suggest that these pigments would play an antioxidant role. In that way, a UV-B UV-A filter function of these carotenoids would be discarding because pigment absorption ranged between 400 and 550 nm (unpublished data). The mechanisms behind the UV resistance of these strains are intriguing and worthy of future research efforts.

Acinetobacter johnsonii A6 was the unique Gram-negative non-spore-forming bacterium found with the culture technique used. Its UV-B resistance performance was similar to that found in most Gram-positive pigmented bacteria. Previous reports have shown the effect of solar UV radiation on viability of Antarctic isolated *Acinetobacter* sp. strain [7]. On the other hand, *Acinet-*

Table 2. D values[a] (kJ m^{-2}) and slopes[b] from survival during total community UV-B exposition

Strain	Slope$^{st\ dev}$	r	D_{37}	D_{90}
Bacillus megaterium A1	$-21.39^{\pm 0.001}$	0.99	2.9	4.2
Acinetobacter johnsonii A2	$-22.03^{\pm 0.36}$	0.99	2.9	4.1
Staphylococcus saprophyticus A3	$-21.39^{\pm 0.20}$	0.99	2.9	4.2
Bacillus pumillus A4	−22.47	1	2.8	4
Nocardia sp. *A5*	$-15.73^{\pm 0.75}$	0.94	4	ND[c]

[a]D values are defined as the UV doses that reduced a cell population to a specific percentage of the original number of cells. The D-values were calculated from the regression line of the exponential slope of the survival curve (D comes from dosis).
[b]Slope calculated in linear relationship from the survival curve.
[c]D_{90} could not be determined because strain A5 never achieves the 90% inhibition during the whole exposure experiment.

obacter sp. strain was the unique Gram-negative bacteria found in spacecraft assembly. It exhibited resistance to desiccation, H_2O_2 exposure, and gamma radiation [11].

The percentage of cells of bacteria belonging to dominant species varied depending on the UV doses. At the same time, the UV irradiation of water made it possible to reveal minor species, demonstrating that its UV-B-resistant phenotype favored its developments and predominance under irradiation conditions.

In this article, it was found that the total number of bacteria and the number of dominant species in water samples were influenced by UV-B irradiation treatment; however, a general and homogeneous resistance was found in most of the community species, indicating that most bacteria that inhabit this environment are well adapted to strong UV-B regimen. In other way, it is proposed that direct UV irradiation of the natural samples can be used as a simple method for the isolation of UV resistant species from the environment.

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