RESEARCH LETTER

UV-resistant *Acinetobacter* sp. isolates from Andean wetlands display high catalase activity

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Andean wetlands; *Acinetobacter*; UV radiation; oxidative stress; superoxide dismutase; catalase.

Abstract

Andean wetlands are characterized by their extreme environmental conditions such as high UV radiation, elevated heavy metal content and salinity. We present here the first study on UV tolerance and antioxidant defense of four *Acinetobacter* strains: Ver3, Ver5 and Ver7, isolated from Lake Verde, and N40 from Lake Negra, both lakes located 4400 m above sea level. All four isolates displayed higher UV resistance compared with collection strains, with Ver3 and Ver7 being the most tolerant strains not only to UV radiation but also to hydrogen peroxide (H_2O_2) and methyl viologen (MV) challenges. A single superoxide dismutase band with similar activity was detected in all studied strains, whereas different electrophoretic pattern and activity levels were observed for catalase. Ver3 and Ver7 displayed 5–15 times higher catalase activity levels than the control strains. Analysis of the response of antioxidant enzymes to UV and oxidative challenges revealed a significant increase in Ver7 catalase activity after H_2O_2 and MV exposure. Incubation of Ver7 cultures with a catalase inhibitor resulted in a significant decrease of tolerance against UV radiation. We conclude that the high catalase activity displayed by Ver7 isolate could play an important role in UV tolerance.

Introduction

Several *Acinetobacter* clinical isolates have been found in the last 40 years causing a high number of severe nosocomial diseases and increasing cases of community-acquired infections, especially in immunocompromised patients (Mussi *et al.*, 2007; Jung *et al.*, 2010; Nemec & Dijkshoorn, 2010; Sullivan *et al.*, 2010). *Acinetobacter baumannii* strains are the most frequently presented in the literature, particularly associated with multidrug resistance, including an emerging resistance to carbapenems (Mussi *et al.*, 2005; Dijkshoorn *et al.*, 2007; Doi *et al.*, 2009). Although they are widely distributed, much less has been investigated about environmental *Acinetobacter* isolates and their impact in water and soil ecosystems (Vanbroekhoven *et al.*, 2004; Kim *et al.*, 2008; Girlich *et al.*, 2010).

Four *Acinetobacter* strains have been isolated recently from the Andean lakes Verde and Negra as part of a collection of more than 200 strains from Andean lakes (Ordoñez *et al.*, 2009). These aquatic ecosystems, named *high-altitude Andean wetlands* (HAAW), are located at more than 4400 m above sea level in the sedimentary-volcanic plateau called Andean Altiplano. Besides high UV radiation, unique features characterize these environments, including high salinity and elevated content of heavy metals, restricting microbial life to those species that are able to tolerate these extreme conditions (Flores *et al.*, 2009).

UVB (280–320 nm) exposure not only provokes photochemical damage of biomolecules but also promotes generation of reactive oxygen species (ROS), eliciting prooxidant imbalance and oxidative stress (Dai *et al.*, 2006; Svobodova *et al.*, 2006). The generated ROS lead to oxidative destruction of cell components through oxidative damage of membrane lipids, nucleic acids and proteins (Shiu & Lee, 2005; Li *et al.*, 2010b).

Living organisms have developed antioxidant strategies to cope with ROS accumulation, including the evolution of enzymatic scavengers such as catalase and superoxide dismutase (SOD), which represent two conspicuous antioxidant enzymes widely distributed in nature (Sies, 1997). We present here the first study on the UVB response and the antioxidant enzymatic defense of *Acinetobacter* HAAW

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isolates Ver3, Ver5 and Ver7 from Lake Verde and N40 from Lake Negra.

Materials and methods

Bacterial strains and culture media

Bacterial strains Ver3, Ver5 and Ver7 were isolated from Andean Lake Verde and strain N40 from Lake Negra (Ordoñez *et al.*, 2009). Both lakes are located at 4400 m above sea level in Catamarca, Argentina. All four strains belong to the Extremophile Strain Collection from the Laboratory of the Andean Lakes Microbiology Research (http://www.limla.com.ar). Three strains from the German Collection of Microorganisms and Cell Cultures (DSMZ) – *A. baumannii* DSM 30007, *Acinetobacter johnsonii* DSM 6963 and *Acinetobacter lwoffii* DSM 2403 – were included in the assays for comparison. *Escherichia coli* DH5α strain was used as a control for *in situ* SOD inhibition assay as described below.

All cultures were grown in Luria–Bertani (LB) broth, supplemented with 2% agar for solid medium when applicable.

Sequence alignments and phylogenetic tree

The 16S rRNA gene sequences from 34 *Acinetobacter* strains used in this work were obtained from the National Center for Biotechnology Information (NCBI), (the corresponding accession numbers are: AM778686.1, AM410706.2, AM778688.1, AF509828.1, AM778690.1, X81663.1, AM778696.1, EU337121.1, AF509825.1, AJ293694.1, AF509826.1, AJ293693.1, GU388381.1, AJ626712.1, NR_028853.1, AJ303013.1, FJ907197.1, AJ295007.1, EU661706.1, FJ608110.1, FJ860867.1, EF204273.1, GQ200824.1, DQ289068.1, FN563422.1, EF204280.1, FN563420.1, GU083586.1, X81660.1, FJ263924.1, AF509827.1, FN393792.1, NR_028851.1 and X81665.1.)

The 16S rRNA gene sequences of the four isolates studied here were amplified previously using universal primers (F-27: AGAGTTTGATAMTGGCTCAG, R-1492: TACGGY TACCTTGTTACGACTT) and sequenced as described (Ordoñez *et al.*, 2009). Nucleotide database searches were performed at NCBI using the BLAST network service.

To construct the phylogenetic trees, the sequences were aligned in the CLUSTAL X 2.0.9 program, which is a Windows interface for the CLUSTAL W multiple sequence alignment program (Larkin *et al.*, 2007). TREEVIEW X version 0.5.0 was used to display phylogenies. All positions containing gaps and missing data were eliminated from the dataset manually. Analyses were performed by the neighbor-joining (NJ) distance method within the same program (Saitou & Nei, 1987). Confidence limits to the inferences obtained by NJ were placed by the bootstrap procedure.

Sensitivity to UV radiation and pro-oxidants in agar plates

Bacterial cultures collected at an $OD_{600\,nm}$ of 0.4 were subjected to serial dilutions. Aliquots of 10 μL were then loaded onto LB agar plates, supplemented with methyl viologen (MV) (0.15 mM) or hydrogen peroxide (H_2O_2) (0.35 mM) when indicated.

To evaluate tolerance to UV, plates were exposed to 9×10^3 J m^{-2} radiation using UVB lamps (maximum emission 302 nm, Bio-Rad Life Science) as light source. The radiation intensity reaching agar surface in disposable Petri plates was measured under the plastic lid using a UVB/UVA radiometer (UV203 AB radiometer; Macam Photometrics). When applicable, cultures were pretreated with 50 mM of the catalase inhibitor 3-amino-1,2,4-triazole (AT) for 60 min.

CFU counting after UVB exposure

Bacteria were aerobically grown in 50 mL of LB broth using 250-mL Erlenmeyer flasks on an orbital shaker (200 r.p.m.) at 30 °C. When cultures reached an $OD_{600\,nm}$ of 0.4, aliquots of 15 mL were exposed to UVB radiation in disposable covered Petri plates (2.0–3.0 W m^{-2} for 30–60 min). Radiation intensity was measured under the plastic lid using the UVB/UVA radiometer described above. After exposure, culture aliquots were subjected to serial dilutions in four replicates and spread onto LB agar plates for later counting of CFU.

Antioxidant enzyme activity

Cells grown to exponential phase ($OD_{600\,nm}$~0.8), were disrupted by sonic oscillation (30 s, Branson Sonifier 250) in 20 mM Tris-HCl containing 5 mM EDTA, 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride and 14 mM β-mercaptoethanol. Lysates were cleared by centrifugation (10 000 ***g***, 15 min) and protein concentration was estimated in the supernatant by a dye-binding assay (Bradford, 1976) using bovine serum albumin as standard.

SOD activity was visualized *in situ* after electrophoresis of the corresponding cellular lysates in nondenaturing polyacrylamide gels as described previously (Beauchamp & Fridovich, 1971), using inhibition by H_2O_2 and KCN to determine the metal identity in the enzyme (Donahue *et al.*, 1997). SOD activity was also determined spectrophotometrically by inhibition of the xanthine/xanthine oxidase-induced reduction of cytochrome *c* at pH 7.8 (McCord & Fridovich, 1969).

Catalase activity was visualized *in situ* after electrophoresis in nondenaturing polyacrylamide gels, as described previously (Scandalios, 1968). Spectrophotometric

measurements were carried out by following the decay of H_2O_2 at 240 nm (Aebi, 1984).

To evaluate the effect of pro-oxidants and UV radiation on the antioxidant activities in the studied strains, cell-free soluble extracts were obtained using the same protocol described above after the challenge was performed.

Results

Fragments of 800 bp of the 16S rRNA genes from the HAAW isolates Ver3, Ver5, Ver7 and N40 were subjected to sequence alignment using the CLUSTAL X 2.0.9 program (Larkin *et al.*, 2007) including 30 available *Acinetobacter* NCBI entries (base 7 to base 821 of *A. baumannii* DSM 30007, accession number X81660.1). Figure 1 shows the resulting unrooted tree after applying the NJ algorithm (Saitou & Nei, 1987).

The Ver5 and N40 isolates clustered together including seven *A. lwoffii* strains. When compared pairwise, Ver5 and N40 strains showed 99.26% of DNA sequence identity between them, and 99.37% and 99.27% with *A. lwoffii* DSM 2403 DNA, respectively (not shown). Although this similarity does not confirm Ver5 and N40 species identity with *A. lwoffii*, it indicates a close phylogenetic relationship among them (Achtman & Wagner, 2008). Ver3 and Ver7 strains presented 98.02% and 97.76% of DNA sequence identity with *A. baumannii* DSM 30007, respectively and, as depicted in Fig. 1, they grouped as a small cluster with a 99.51% of identity between them. These values suggest that Ver3 and Ver7 belong to a different species than *A. baumannii* DSM 30007 (Achtman & Wagner, 2008).

Results of Gram staining, motility and cytochrome *c* oxidase classical assays (Schreckenberger & von Graevenitz, 1999) also fit *Acinetobacter* genus for all four isolates (not shown). Only Ver3 and Ver7 strains grew at 44 °C in LB medium, as described for the *A. baumannii–calcoaceticus* group (Schreckenberger & von Graevenitz, 1999). In this work, *A. baumannii* DSM 30007, *A. johnsonii* DSM 6963 and *A. lwoffii* DSM 2403 were used as control strains.

Tolerance to UV radiation was tested by placing culture serial dilutions drops of the studied strains on LB agar plates and exposing to UV source as described (see Materials and methods). Our results showed that all four HAAW isolates were more resistant to radiation than were selected control strains (Fig. 2). Ver3 and Ver7 were the most tolerant strains, being able to grow even after 60 min of exposure to 2.6 W m^{-2} UVB radiation.

Similar protocols were performed to evaluate tolerance to oxidant agents, using culture media supplemented with MV or H_2O_2 to challenge *Acinetobacter* strains. Once inside the cell, MV is enzymatically reduced and promotes the generation of superoxide functioning as a radical propagator (Carr *et al.*, 1986). H_2O_2 is a weak oxidant itself, although it is able to cause severe damage through its conversion to hydroxyl radical via Fenton reaction (Imlay, 2003), rapidly reacting with most cell biomolecules, including lipids, amino acids and nucleic acids. In contrast to the observed behavior under UV exposure, the response of N40 and Ver5 isolates was similar to that of the control strains when challenged with

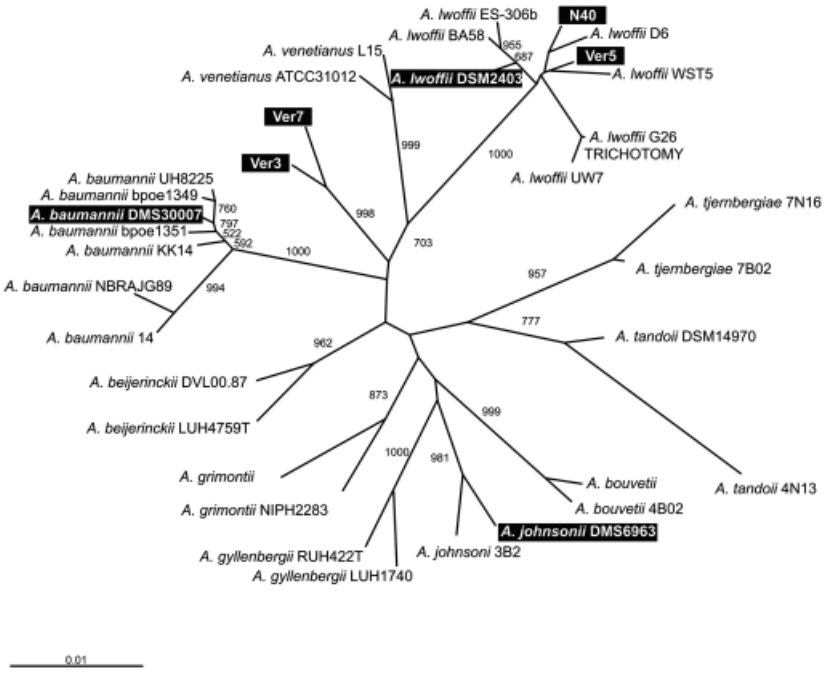


Fig. 1. Unrooted phylogenetic tree. Phylogenetic tree generated using the NJ method for *Acinetobacter* strains including reference sequences from GenBank (see Materials and methods). Robustness of the major branching points is indicated by the bootstrap values from NJ analysis. Isolates and control strains used in this work are indicated. Trichotomy designates a point where the chronological order of the groups involved is unclear or unknown.

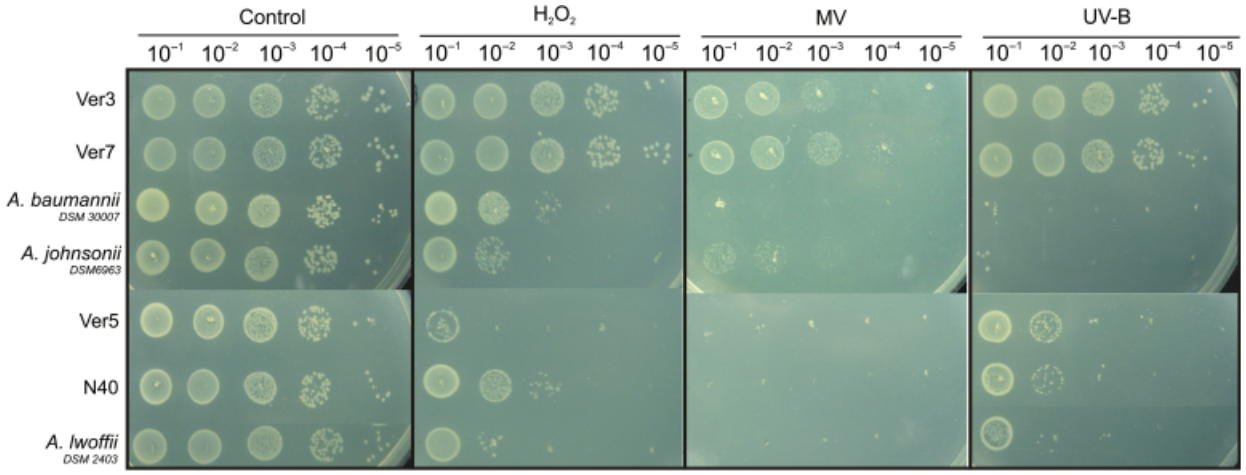


Fig. 2. Sensitivity to UV radiation and pro-oxidants in agar plates. To investigate tolerance against UV radiation and pro-oxidants, aliquots of 10 μL from culture serial dilutions from $OD_{600\,nm}$∼0.4 were loaded onto LB agar plates. When indicated, oxidative agents were added (0.35 mM H_2O_2, 0.15 mM MV). UVB tolerance was examined after exposure of the culture drops to 9×10^3 J m^{-2}. Serial dilutions are indicated at the top and strains identity is specified on the left.

H_2O_2; Ver3 and Ver7 were always the most tolerant strains (Fig. 2). When 0.15 mM MV was present in the culture media, only Ver3 and Ver7 isolates were able to grow at the 10^{-3} dilution. No growth was observed for the rest of the studied strains at the tested conditions, with the exception of a very limited growth of *A. johnsonii* DSM 6963 (Fig. 2).

SODs and catalases are central enzymatic antioxidant scavengers and could be responsible of differential response to oxidative stress among bacteria. A single SOD activity was visualized in polyacrylamide gel electrophoresis (PAGE) in all seven *Acinetobacter* studied strains (Fig. 3a–c). The SOD electrophoretic band was inhibited by 2 mM H_2O_2 but was not sensitive to KCN, behaving as an Fe-SOD enzyme, although a cambialistic SOD should not be disregarded (Fig. 3a–c). Activity measured spectrophotometrically in soluble extracts (see Materials and methods), was between 50 and 100 U mg^{-1} for all studied strains (Fig. 3e).

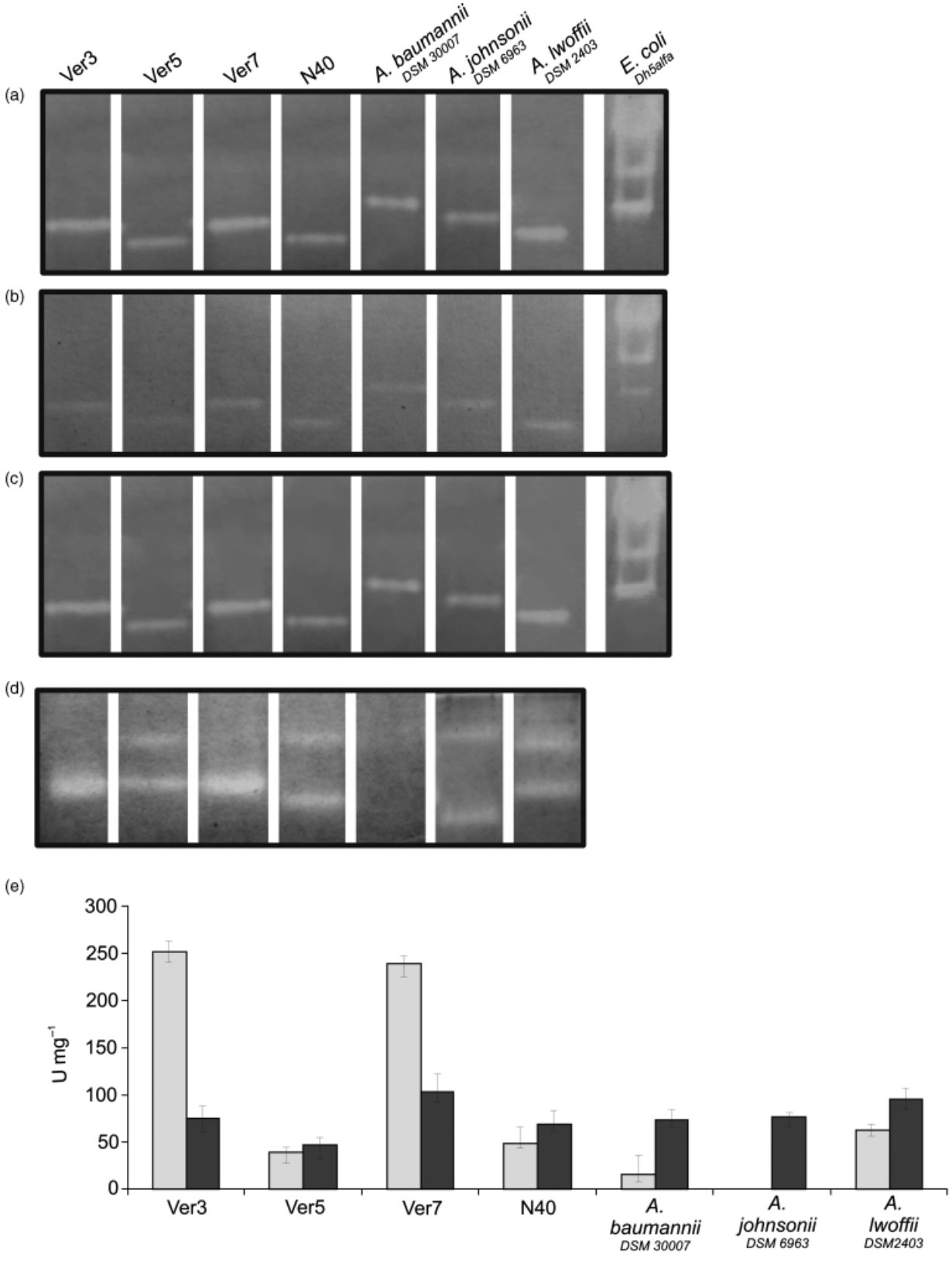
In contrast, the electrophoretic activity pattern and spectrophotometric measurements of catalase diverged among strains. Ver3 and Ver7 soluble extracts showed a single catalase electrophoretic band, whereas Ver5, N40, *A. baumannii* DSM 30007 and *A. lwoffii* DSM 2403 showed two activity bands after native PAGE (Fig. 3d). Interestingly, the activity levels measured spectrophotometrically in Ver3 and Ver7 extracts were 5–15 times higher than those corresponding to the control strains (Fig. 3e). Intriguingly, no catalase activity was detectable in *A. johnsonii* DSM 6963 soluble extracts.

Taking into account that Ver7 displayed the highest tolerance to UV and pro-oxidants among the studied strains (Fig. 2), survival measurements were carried out using *A. baumannii* DSM 30007 as control. As expected from the assays described in Fig. 2, Ver7 showed no significant decrease in CFU when liquid cultures were exposed to 10 kJ m^{-2} of UVB radiation (Fig. 4). In contrast, *A. baumannii* DSM 30007 survival decreased after 30 min, reaching 5% of the control CFU count after 60 min of challenge (Fig. 4).

To evaluate the effect of oxidants and UV radiation on the antioxidant cell response, enzymatic activities were determined before and after challenges. Exposure to 2.5 mM H_2O_2 increased the catalase activity 50–100% in both *A. baumannii* DSM 30007 and Ver7 strains (Fig. 5). Incubation of bacteria in the presence of 2.5 mM MV reduced catalase in *A. baumannii* DSM 30007, whereas this enzymatic activity increased up to 100% in the Ver7 isolate. SOD activity was not affected by MV or H_2O_2 in either Ver7 or control strain *A. baumannii* DSM 30007 (Fig. 5).

Exposure to UV radiation caused no significant variation in SOD activity. However, a 50% decrease in catalase activity was observed for *A. baumannii* DSM 30007 after 60 min of UVB exposure, whereas Ver7 isolate catalase hardly diminished after treatment (Fig. 5).

Fig. 3. SOD and catalase activity measurements. Aliquots of soluble cell extracts of the indicated strains corresponding to ∼10 μg of protein were resolved by nondenaturating PAGE (a–d) and subsequent *in situ* staining for SOD (a–c) or catalase (d) activity. The inhibitory effect of 2 mM H_2O_2 (b) or 2 mM KCN (c) on SOD activity was also evaluated. Soluble enzymatic activities were measured spectrophotometrically (e) on exponential culture extracts ($OD_{600\,nm}$∼0.8), following the protocols described in Materials and methods. Catalase activity is depicted as light gray bars and SOD is shown as dark gray bars (e). No catalase activity was detected in *Acinetobacter johnsonii* DSM 6963 soluble extracts.



AT has been described as a inhibitor of catalase/hydroperoxidase I (Havir, 1992). When Ver7 cells exposed to UVB radiation were pretreated with 50 mM AT, a significant decrease of resistance was observed (Fig. 6).

Discussion

In this work, we studied the antioxidant defense and UV tolerance of four *Acinetobacter* environmental isolates. Using 800-bp fragments of the 16S rRNA genes we constructed an alignment and a phylogenetic tree, finding a well-defined localization of Ver5 and N40 in *A. lwoffii* group, while Ver3 and Ver7 clustered closer to *A. baumannii* strains (Fig. 1).

According to our observations, all four isolates presented more resistance to UVB exposure compared with the control collection strains, although they displayed diverse responses to challenges against oxidant agents (Fig. 2). Interestingly, Ver3 and Ver7 showed the higher tolerance not only to UVB but also to H_2O_2 and MV (Fig. 2).

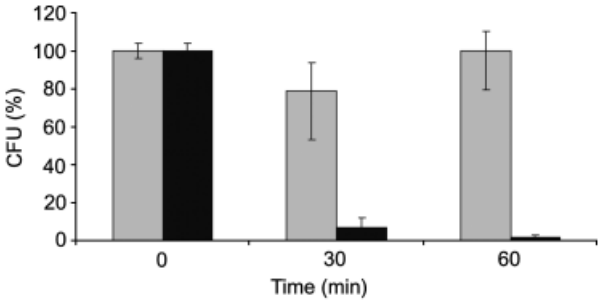


Fig. 4. CFU counting after UVB exposure. Cells were grown to exponential phase ($OD_{600\,nm}$~0.4) and exposed to 2.0–3.0 W m^{-2} of radiation intensity for 30–60 min. After treatment, cells were subjected to serial dilutions in four replicates and spread onto LB agar plates for counting. Percentage of CFU was depicted as gray bars for Ver7 isolate and black bars for *Acinetobacter baumannii* DSM 30007 strain. The 100% corresponds to $1.57 \pm 0.11 \times 10^7$ CFU for *A. baumannii* DSM 30007 and $2.03 \pm 0.23 \times 10^7$ CFU for Ver7 isolate.

Catalase measurements also exhibited differences among strains. Ver3 and Ver7 isolates showed a single band corresponding to activity levels 5–15 times higher than the control strains, which displayed two catalase bands in PAGE. The absence of detectable catalase activity in *A. johnsonii* DSM 6963 soluble extracts is quite intriguing as *Acinetobacter* strains have been described as catalase-positive bacteria in previous reports (Schreckenberger & von Graevenitz, 1999). A particularly unstable protein could have been formed in this case, as it was the only nondetectable catalase among all seven studied extracts, which were all disrupted following the same protocol (see Materials and methods).

In contrast to the divergence found for catalase activity, a single Fe-SOD band was visualized in all seven strains displaying similar spectrophotometric activity values. These results suggest the existence of a variety of complex tolerance mechanisms among *Acinetobacter* strains rather than a common defense pathway for the whole genus.

Previous investigations tried to ascertain a relationship between UV response and antioxidant enzyme activities in bacteria attaining divergent conclusions. Soung & Lee (2000) reported a surprisingly high catalase activity in the radioresistant *Deinococcus* sp. strains. Moreover, an insertional mutant in *kat*A gene of *Deinococcus radiodurans* was shown to be more sensitive to ionizing radiation than the wild-type strain (Markillie *et al.*, 1999). However, *E. coli kat*E and *kat*G single mutants displayed hardly any decrease of survival after near-UV radiation treatment, suggesting a minor role for catalase in UV protection in enterobacteria (Eisenstark & Perrot, 1987). More concluding observations implied SOD participation in the UV defense, as *E. coli sod*A *sod*B double mutants suffered an increase in near-UV

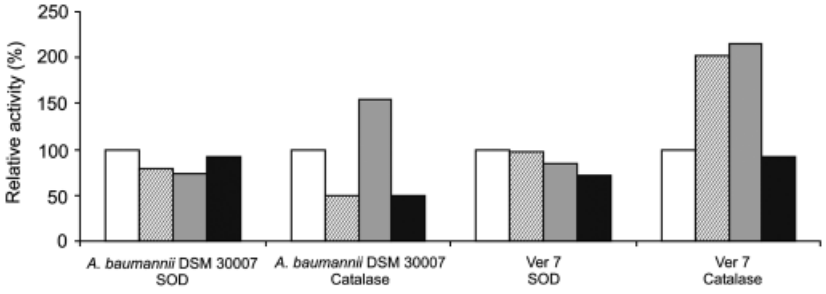


Fig. 5. Effect of oxidant agents and UVB radiation on antioxidant enzymes. Cultures of Ver7 and *Acinetobacter baumannii* DSM 30007 strains were grown to exponential phase ($OD_{600\,nm}$~0.8) and divided into equivalent aliquots. Cultures were then incubated for 60 min at 30 °C without addition (white bars) or supplemented with 2.5 mM MV (lined bars) or 2.5 mM H_2O_2 (gray bars). The 100% catalase activity of soluble extracts corresponds to 12.9 U mg^{-1} for *A. baumannii* DSM 30007 and 138.8 U mg^{-1} for the Ver7 isolate, while control levels for SOD activity were 41.5 U mg^{-1} for *A. baumannii* DSM 30007 and 62.9 U mg^{-1} for the Ver7 isolate. To test the UV effect, 20-mL aliquot of exponentially grown cells ($OD_{600\,nm}$~0.8) placed in covered disposable Petri dishes were exposed to 9×10^3 J m^{-2} of UVB radiation (black bars) or kept in the dark to obtain the 100% control level (white bars). Catalase activities in soluble extracts corresponding to nonexposed cells were 37.4 U mg^{-1} for *A. baumannii* DSM 30007 and 362.8 U mg^{-1} for the Ver7 isolate, while SOD activities were 57.2 U mg^{-1} for *A. baumannii* DSM 30007 and 95.5 U mg^{-1} for the Ver7 isolate.

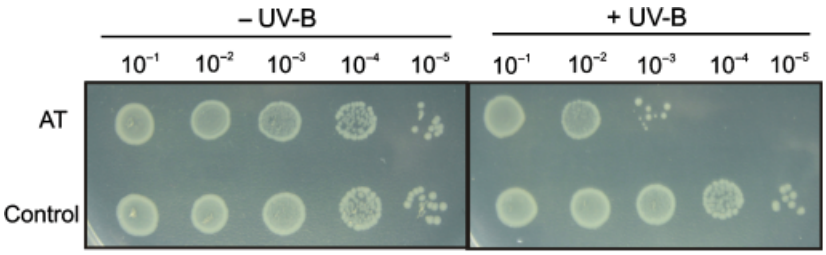


Fig. 6. Effect of catalase on UVB tolerance. Exponential cultures ($OD_{600\,nm}$~0.4) were incubated at 30 °C in an orbital shaker without additives (control) or supplemented with 50 mM AT. After 60 min, cells were collected, washed with fresh LB broth and subjected to serial dilutions (indicated at the top). Aliquots of 10 μL were applied onto LB agar plates and exposed to UVB radiation (9×10^3 J m^{-2}).

sensitivity compared with the wild-type strain (Knowles & Eisenstark, 1994).

Ver3 and Ver7 isolates, with the highest catalase activity among all seven studied strains (Fig. 3d and e), displayed a good tolerance to the pro-oxidants assayed (Fig. 2) and, interestingly, the highest resistance to UV radiation (Fig. 2). Based on our results, a correlation among high catalase activity, H_2O_2 tolerance and UVB radiation resistance could be inferred. Moreover, inhibition of catalase by AT resulted in a decrease of the observed tolerance to UV radiation by Ver7 *Acinetobacter* strain (Fig. 6). Indeed, catalase has an important role in UV defense but, taking into consideration the complexity of the protection response, it seems not to be the only actor playing the scene. The involvement of light-dependent DNA repair systems in the defense machinery against UV radiation has been suggested (Fernandez Zenoff *et al.*, 2006). The presence of photolyase activities able to repair UV-provoked DNA damage in a blue light-dependent manner (Weber, 2005; Li *et al.*, 2010a) is currently under research in HAAW isolates (V. H. Albarracin & M. E. Farías, pers. commun.). Recently, a study has been published reporting that the *phr*A gene encoding a photolyase in *Rhodobacter sphaeroides* is upregulated by singlet oxygen and by H_2O_2 signals involving a σ E factor, and proposing a coordinate regulation between both UV and the antioxidant defense system (Hendrischk *et al.*, 2007).

When we evaluated the effect of pro-oxidants and UVB exposure on antioxidant enzymes, the Ver7 isolate and *A. baumannii* DSM 30007 strain displayed different responses to challenges (Fig. 5), suggesting dissimilar regulatory mechanisms. Catalase activity increased up to 100% in the Ver7 isolate after MV and H_2O_2 treatment, whereas *A. baumannii* DSM 30007 showed no positive response in the same conditions. In addition, Ver7 antioxidant enzymes seem to be less sensitive to UVB exposure than those of the control strain (Fig. 5), reinforcing the idea that the *Acinetobacter* strains exhibit diverse defense strategies to deal with radiation or oxidative challenges. With the exception of an ORF homologue to *oxyR* found in *A. baumannii* sp. ADP1 (Geissdorfer *et al.*, 1999), which encodes a H_2O_2 response regulator (Storz *et al.*, 1990), little is known about *A. baumannii* antioxidant metabolism and adaptive responses. Taking advantage of the available genome sequence of *A. baumannii* ATCC 17978 (Smith *et al.*, 2007), a proteomic study has been recently published suggesting the presence of robust antioxidant machinery in this species (Soares *et al.*, 2010); however, no functional studies of this have been reported.

In this study, we found unusually high catalase activity in the strongly UV-tolerant Ver3 and Ver7 *Acinetobacter* isolates. Moreover, the use of a specific inhibitor suggested the involvement of this enzyme in the resistance against UV radiation. These results provide the basis for further research on the molecular strategies displayed by these isolates to endure the extreme environmental conditions of HAAW.

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