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ORIGINAL PAPER

Wavelength dependence of biological damage induced by UV radiation on bacteria

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Abstract The biological effects of UV radiation of different wavelengths (UVA, UVB and UVC) were assessed in nine bacterial isolates displaying different UV sensitivities. Biological effects (survival and activity) and molecular markers of oxidative stress [DNA strand breakage (DSB), generation of reactive oxygen species (ROS), oxidative damage to proteins and lipids, and the activity of antioxidant enzymes catalase and superoxide dismutase] were quantified and statistically analyzed in order to identify the major determinants of cell inactivation under the different spectral regions. Survival and activity followed a clear wavelength dependence, being highest under UVA and lowest under UVC. The generation of ROS, as well as protein and lipid oxidation, followed the same pattern. DNA damage (DSB) showed the inverse trend. Multiple stepwise regression analysis revealed that survival under UVA, UVB and UVC wavelengths was best explained by DSB, oxidative damage to lipids, and intracellular ROS levels, respectively.

Keywords UV radiation · Bacteria · Inactivation · Oxidative stress

Introduction

Bacteria are very susceptible to the effects of UV radiation, due to their small size, short generation time and absence of effective UV-protective pigmentation (Garcia-Pichel 1994). Bacterial isolates have different susceptibilities to UV radiation (Joux et al. 1999; Arrieta et al. 2000; Berney et al. 2006c; Chun et al. 2009; Santos et al. 2011, 2012a), and UV sensitivity is dependent on the wavelength (Sundin and Jacobs 1999; Qiu et al. 2004; Bauermeister et al. 2009). Since different biomolecules (e.g., DNA, proteins and lipids) absorb UV radiation at different wavelengths, a portion of this variability is likely due to differences in the preferable cellular targets of the various wavelengths. DNA is considered the major target of UV radiation. However, comparable levels of DNA photoproduct accumulation are observed in bacteria displaying different sensitivities to UV radiation (Joux et al. 1999; Matallana-Surget et al. 2008). In addition, DNA damage alone cannot account for the inhibition of bacterial activity in surface waters (Visser et al. 2002). Accordingly, it is likely that damage to other biomolecules contributes to the inhibitory effects of UV radiation and the variation in UV sensitivity among bacterial isolates.

UV radiation can be divided into three regions: UVA (320–400 nm), UVB (280–320 nm) and UVC (10–280 nm). UVC is filtered by the ozone layer (O_3) and does not reach the Earth's surface. Terrestrial radiation, often called sunlight, contains about 8 % UVA and less than 1 % UVB (Coohill and Sagripanti 2009). The biological effects of UVA are usually attributed to enhanced production of reactive oxygen species (ROS), which results in oxidative damage to lipids, proteins and DNA (Chamberlain and Moss 1987; Moan and Peak 1989; Girotti 1998; Pattison and Davies 2006; Zeeshan and Prasad 2009). UVB and UVC photons cause direct DNA damage by inducing the formation of DNA lesions (photoproducts), most notably pyrimidine dimers, which block DNA replication and RNA transcription (Pfeifer 1997). Exposure to UVB also causes oxidative stress, as evidenced

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by the expression of antioxidant defenses following UVB irradiation (Qiu et al. 2005; Matallana-Surget et al. 2009a). Although the UVC region is not environmentally relevant, it is useful for assessing the UV sensitivity of bacteria that are highly tolerant or insensitive to high doses of UVB (Sundin and Jacobs 1999). UVC radiation is also well known for its bactericidal potential (Jagger 1985; Coohill and Sagripanti 2008; King et al. 2011).

Most studies addressing the cellular effects of UV radiation on bacteria conducted to date have focused on one target (either proteins, lipids or DNA) in one bacterial strain (Abboudi et al. 2008; Matallana-Surget et al. 2008, 2009a; Bosshard et al. 2010b), which has prevented a full understanding of the molecular basis for the variability in UV sensitivity. Such information is crucial to understand the role of UV radiation as a driver of microbial diversity and function in ecosystems, as well as to the development of efficient and ecologically-friendly UV-based disinfection strategies targeting a broad range of bacteria.

The objective of this study was to identify the major determinants of cell inactivation under different UV wavelengths in a set of bacterial isolates displaying distinct UV sensitivity.

Materials and methods

Experimental layout

The bacterial isolates used in this study were previously isolated from the surface waters of the estuarine system of Ria de Aveiro (Portugal) and characterized in terms of UV sensitivity (Santos et al. 2011) (Table 1). In all irradiation experiments, only vegetative cells were used.

Fresh bacterial cultures were prepared in Marine Broth 2216 (Difco, Detroit, MI) and grown with agitation (120 rpm) at 25 °C. Cells were harvested by centrifugation (3,200×*g* for 15 min) in the late exponential phase (defined as the inflection point of the growth curve, at the transition between the exponential and stationary phase, which was usually achieved in 8–14 h). The growth rates (μ) of the isolates were determined as previously described (Berney et al. 2006b) as $\mu = \Delta \ln OD_{546}/\Delta t$. The pellet was washed three times in 0.2-µm-pore-size-filtered autoclaved 0.9 % NaCl solution and cells were resuspended in filter-sterilized autoclaved 0.9 % NaCl. Bacterial abundance was determined by epifluorescence microscopy after acridine orange staining (Hobbie et al. 1977), adjusted with filtered autoclaved 0.9 % NaCl to 10^6 cells mL^{-1} and homogenized by gentle vortexing.

For each isolate, 30 mL of bacterial cell suspension (corresponding, on average, to a biomass of approximately 1 mg mL^{-1} of protein) was transferred to sterile 150 × 25 mm plastic tissue culture dishes (Corning Science Products, Corning, NY, USA) so that the depth of the liquid was <2 mm. For irradiation, the lid was removed and cell suspensions were exposed to UVA (Philips TL 100 W/10R lamps, Philips, Eindhoven, The Netherlands, main emission line at 365 nm, intensity of 50 W m^{-2}), UVB (Philips TL 100 W/01 lamps, Philips, Eindhoven, The Netherlands, main emission line at 302 nm, intensity of 2.3 W m^{-2}) and UVC (low pressure mercury lamp NN 8/15, Heraeus, Berlin,

Table 1 Bacterial strains used in the experiments, their accession number, phylogenetic affiliation, closest relatives, similarity with database, as well as bacterial group and growth rates

Strain	Accession number	Phylogenetic affiliation	Closest relative in database (accession number)	% 16S rDNA similarity	Growth rate, μ (h^{-1})	UVA LD_{50} (kJ m^{-2})	UVB LD_{50} (kJ m^{-2})	UVC LD_{50} (J m^{-2})
PT5I1.2G	GQ365202	*Acinetobacter* sp.	*Acinetobacter sp.* (EU545154.1)	99	0.3	159.3	34.1	18.0
PT15I3.2CB	GQ365209	*Bacillus* sp.	*Bacillus thuringiensis* (JN084031.1)	99	0.7	152.7	34.1	31.2
PT5I3.3L	GQ365205	*Brevibacterium* sp.	*Brevibacterium sp.* (JF905605.1)	99	0.3	51.9	38.1	16.4
NT25I3.2AA	GQ365196	*Micrococcus* sp.	*Micrococcus* sp. (HM352362.1)	98	0.4	297.5	50.1	45.0
NT25I3.1A	GQ365195	*Paracoccus* sp.	*Paracoccus* sp. (AB681547.1)	99	0.4	285.3	40.3	13.7
NT5I1.2B	GU084169	*Pseudomonas* sp.	*Pseudomonas* sp. (JF749828.1)	99	0.7	221.3	49.3	40.6
PT15I3.2CA	GQ365208	*Psychrobacter* sp.	*Psychrobacter piscidermidis* (EU127295.1)	99	0.1	228.2	29.6	16.7
NT15I1.2B	GU084171	*Sphingomonas* sp.	*Sphingomonas* sp. (AM900788.1)	100	0.3	290.9	39.2	40.6
NT25I2.1	GQ365197	*Staphylococcus* sp.	*Staphylococcus saprophyticus* (HQ407261.1)	99	0.3	138.1	37.0	17.4

LD_{50} values for each strain under the different UV spectral regions are also shown. Note that UVA and UVB doses are expressed in kJ m^{-2} while UVC doses are expressed in J m^{-2}

Germany, main emission line at 254 nm, intensity of 0.66 W m^{-2}). UV sources were placed at 20 cm from the sample. UV intensities were measured with a monochromator spectro-radiometer placed at the sample level (DM 300, Bentham Instruments, Reading, UK), and the UV dose (in J m^{-2}) was calculated by multiplying the intensity by the irradiation time (in seconds). The total cumulative doses applied were 300 kJ m^{-2}, 90 kJ m^{-2} and 180 J m^{-2}, for UVA, UVB and UVC, respectively. During irradiation, samples were stirred by magnetic agitation and temperature was kept at ±25 °C. A dark control (covered in aluminum foil) treated in the same way as the irradiated samples was included in every experiment. Survival curves for each isolate were generated separately for UVA, UVB and UVC for the determination of the LD_{50} (UV dose resulting in 50 % inactivation). Aliquots of cell suspensions were collected before irradiation and at LD_{50} for assessment of activity, indicators of oxidative damage and activity of antioxidant enzymes. Samples were immediately placed at 4 °C in order to avoid repair until further processing, which was generally conducted in less than 1 h. All experiments were repeated in three independent assays. Parameters were always determined in a minimum of three analytical replicates. Positive (50 mM H_2O_2-treated) and negative (untreated) controls were always included and processed along experimental samples in order to ensure proper functioning of the procedures on all strains. All determinations were carried out in a red-dark room to minimize photoreactivation.

Colony forming units (CFU)

Sample aliquots of irradiated samples and non-irradiated controls were serially diluted in filter-sterilized, autoclaved 0.9 % NaCl, and 100 µL aliquots were spread-plated in agar plates (Difco). Colonies were counted after 3 days of incubation in the dark at 25 °C.

Bacterial activity

Bacterial activity was estimated from the rates of [^{3}H] leucine incorporation (Smith and Azam 1992) in cell suspensions before and after UV exposure. Triplicate 1.5 mL aliquots and a trichloroacetic acid (5 %)-fixed control were incubated with a mixture of [^{3}H] leucine (Amersham Biosciences, specific activity 160 Ci $mmol^{-1}$) and non-radioactive leucine at a previously determined saturating concentration of 480 nM. Samples were incubated in the dark at in situ temperatures for 1 h. Incubations were stopped by the addition of TCA (5 % final concentration), after which samples were centrifuged at 16,000×*g* for 10 min. The supernatant was discarded, and 1.5 mL of 5 % TCA was added. The samples were then vortexed and centrifuged, and the supernatant was discarded. The pellet was washed with 90 % ethanol, dried overnight at room temperature and resuspended in 1.0 mL of Universol liquid scintillation cocktail (ICN Biomedicals). The radioactivity incorporated in bacterial cells was measured after 3 days in a Beckman LS 6000 IC Liquid Scintillation Counter.

Intracellular ROS generation

Intracellular production of ROS was detected using the probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Pérez et al. 2007). Control and irradiated samples were centrifuged and washed with 10 mM potassium phosphate buffer (pH 7.0), amended with the probe (final concentration 10 µM), and incubated for 30 min in the dark. Cells were subsequently washed and sonicated, and 100 µL of the cell extracts were mixed with 1 mL of the potassium phosphate buffer. The fluorescence of the samples was measured with a Jasco FP-777 Fluorometer at room temperature, with an excitation wavelength of 490 nm and emission wavelength of 519 nm. The fluorescence intensity at 519 nm was corrected against blank controls without cells and then normalized to the protein content (see below for procedure) in comparison with control samples.

DNA strand breakage

UV-induced DNA damage was assessed using the quantification of DNA strand breaks (DSB) as a proxy, due to the inability to detect the more routinely used CPDs in some of the strains even after exposure to 180 J m^{-2} of UVC, potentially due to experimental constraints. On the other hand, DSB accumulation, as well as the variation of the other parameters assessed, generally followed a dose-dependent trend (data not shown).

DSB was determined following a modified version of the FADU (Fluorimetric Analysis of DNA Unwinding) method (He and Häder 2002). In addition to the test samples (so-called *P*-samples), the method requires two sets of untreated control samples: samples not subjected to alkaline unwinding (*T*-samples) and samples subjected to complete alkaline unwinding (*B*-samples). Cells were collected by centrifugation (3,000×*g*, 15 min) and digested with lysozyme (4 mg mL^{-1} final concentration) in EDTA solution, followed by proteinase *K* (0.25 mg mL^{-1} final concentration). A volume of 300 µL of 0.1 M NaOH was added to the three sets of samples: (1) *T*-samples were neutralized with 300 µL of 0.1 M HCl and sonicated for 15 s, following a 30-min incubation at room temperature, (2) *B*-samples were sonicated for 2 min, neutralized with 300 µL of 0.1 M HCl after a 30-min incubation and sonicated again for 15 s, and (3) *P*-samples were incubated for

30 min, neutralized with 300 µL of 0.1 M HCl and sonicated for 15 s.

A final concentration of 5 µM of Hoechst 33258 was added to all samples and, after centrifugation, a 1 mL volume of supernatant was used for fluorescence measurements (λ_{ex}. 350 nm; λ_{em}. 450 nm) in a Jasco FP-777 Fluorometer. The fraction of double stranded DNA (dsDNA) was calculated as dsDNA = $(P–B)/(T–B) \times 100$, where T, P and B were fluorescence intensities of T-, P- and B-samples normalized to the protein content, respectively.

Thiobarbituric acid reactive substances

Lipid peroxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) as previously described (Pérez et al. 2007). Control and irradiated cells were centrifuged, washed and resuspended in 1 mL of 50 mM potassium phosphate buffer (pH 7.4) added of 0.1 mM butylated hydroxytoluene and 1 mM PMSF (phenylmethanesulfonyl fluoride). After sonication and centrifugation to remove cellular debris, the soluble fraction was mixed with 1 mL of 20 % trichloroacetic acid and centrifuged (10,000×g for 5 min). Supernatants were removed and mixed with 1 mL of 0.5 % (w/v) thiobarbituric acid in 0.1 M HCl and 10 mM butylated hydroxytoluene. Samples were heated at 100 °C for 1 h, after which 1 ml aliquots were removed, cooled and then mixed with 1.5 mL of butanol. After centrifugation (4,000×g, 10 min), the organic fraction was removed and the absorbance at 535 nm was determined using a Thermo Spectronic Genesys 10 UV spectrophotometer. TBARS content was determined using an extinction coefficient of 156 mM cm^{-1}, and values were normalized to the protein content.

Protein oxidation

Protein oxidation was assessed from carbonyl levels as previously described (Semchyshyn et al. 2005). Aliquots of cell homogenates were incubated for 1 h at room temperature with 10 mM dinitrophenylhydrazine (DNPH) in 2 M HCl. DNPH was omitted in the blanks. Proteins were precipitated with 500 µL of 20 % trichloroacetic acid and centrifuged (14,000×g, 5 min), and the pellet was washed three times with 1 mL of 1:1 (v/v) ethanol–ethyl acetate. The final precipitate was dissolved in 1 mL of 6 M guanidine hydrochloride. Samples were spectrophotometrically analyzed against a blank of 1 mL of guanidine solution (6 M guanidine hydrochloride with 2 mM potassium phosphate). The absorbance at 360 nm was determined, and the molar absorption coefficient of 22 mM cm^{-1} was used to quantify the levels of protein carbonyls. Values were normalized to the protein content.

Antioxidant enzymatic activity

Irradiated and non-irradiated cells were resuspended in cold 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and sonicated in ice. The extracts were centrifuged (10,000×g, 15 min) and the supernatant frozen at −80 °C until analysis.

Catalase (CAT) activity was measured spectrophotometrically by monitoring the rate of decomposition of H_2O_2 (Beers and Sizer 1952). One unit of CAT activity was defined as the amount of activity required to decompose 1 µmol of H_2O_2 per minute under the assay conditions. The strain *Enterococcus faecalis* was used as a negative control. An additional negative control consisting of a mixture of 18 mM hydrogen peroxide with sterile potassium phosphate buffer (1:5) was also included in every experiment (Anderl et al. 2003). Superoxide dismutase (SOD) activity was determined according to McCord and Fridovich (1969) in which a xanthine–xanthine oxidase system is used to generate O_2^- and nitroblue tetrazolium is used as an indicator. One unit of SOD activity was defined as the amount of SOD that resulted in 50 % inhibition of the reduction of nitroblue tetrazolium. Potassium phosphate buffer was used as a blank. Protein concentration in cell suspensions was determined by the method of (Bradford 1976). The specific activity of antioxidant enzymes was expressed as units per milligram of cellular proteins.

Statistical analysis

Differences between treatments were assessed by 1-way ANOVA using the statistical software SPSS v.17. Levene test was used to assess homogeneity of variances. If variances were not homogeneous, the non-parametric Mann–Whitney test was used to assess the overall effect of treatment. Differences with p values <0.05 were considered statistically significant. Principal component analysis (PCA), used to reduce the variability of the data sets and identify the main parameters contributing to the discrimination between UV treatments, was performed using software Primer 5. Stepwise multiple regression, used to identify groups of independent variables that would predict the dependent variable (LD_{50}) with optimal efficiency, was conducted on SPSS v. 17.

Results

UV effects on survival and activity

UV sensitivity curves of the isolates under the different spectral regions are shown in Fig. 1 and were used to determine LD_{50} values (Table 1). LD_{50} values showed

expected wavelength dependence, being highest under UVA and lowest under UVC. LD_{50} values for UVA ranged from 51.9 ± 3.7 kJ m^{-2} in *Staphylococcus* sp. to 297.5 ± 16.3 kJ m^{-2} in *Micrococcus* sp. Under UVB, LD_{50} values ranged between 29.6 ± 1.5 kJ m^{-2} in *Psychrobacter* sp. and 50.1 ± 3.8 kJ m^{-2} in *Micrococcus* sp. and for UVC LD_{50} values varied between 13.7 ± 0.3 J m^{-2} in *Acinetobacter* sp. and 45.0 ± 2.4 J m^{-2} in *Micrococcus* sp.

Inhibition of activity also showed a clear wavelength dependence, with UVC wavelengths causing the highest average inhibition (77.5 ± 2.1 %) and UVA the lowest (30.0 ± 20.2 %). For UVA, at LD_{50} inhibition of activity ranged from 5.1 ± 0.1 % in *Pseudomonas* sp. to 56.8 ± 7.2 % in *Psychrobacter* sp. Exposure to LD_{50} of UVB resulted in an inhibition of activity ranging between 38.9 ± 3.4 % in *Sphingomonas* sp. and 80.6 ± 7.6 % in *Bacillus* sp. Under UVC, exposure to LD_{50} resulted in an inhibition of activity ranging between 72.7 ± 6.5 % in *Sphingomonas* sp. and 79.4 ± 7.0 % in *Staphylococcus* sp. (Fig. 2).

ROS generation and oxidation of biomolecules

ROS generation also followed a wavelength trend of variation, being highest under UVA (42.1 ± 9.4 %) and lowest under UVC (8.2 ± 4.1 %). The enhancement of ROS generation was more marked in *Staphylococcus* sp. with UVA (56.4 ± 5.9 %) and UVB (39.1 ± 3.9 %) and *Micrococcus* sp. (15.2 ± 1.8 %) with UVC (Fig. 3a).

The increase in DNA strand breaks (DSB) showed a wavelength dependence as well, being highest under UVC (23.2 ± 8.4 %) and lowest under UVA (7.9 ± 5.7 %). DSB generation ranged from 1.4 ± 0.1 % (*Bacillus* sp.) to 19.0 ± 2.0 % (*Staphylococcus* sp.) under UVA, from 5.3 ± 0.6 % (*Micrococcus* sp.) to 31.3 ± 3.1 % (*Staphylococcus* sp.) under UVB and from 10.7 ± 0.9 % (*Acinetobacter* sp.) to 35.7 ± 4.2 % (*Psychrobacter* sp.) under UVC (Fig. 3b).

The increase in TBARS, used as proxies for the extent of oxidative stress damage to the membrane lipids, varied from 37.1 ± 4.4 % (*Pseudomonas* sp.) to 54.2 ± 5.3 % (*Psychrobacter* sp.) with UVA, from 22.0 ± 2.5 % (*Brevibacterium* sp.) to 36.2 ± 3.5 % (*Bacillus* sp.) with UVB and from 6.3 ± 0.5 % (*Pseudomonas* sp.) to 14.7 ± 1.5 % (*Staphylococcus* sp.) with UVC (Fig. 3c). A wavelength trend of variation was also observed for TBARS, which showed the strongest generation under UVA (47.7 ± 6.2 %) and the lowest under UVC (11.4 ± 3.5 %).

The increase in protein carbonyl levels ranged from 15.2 ± 1.6 % (*Bacillus* sp.) to 97.0 ± 11.5 % (*Sphingomonas* sp.) under UVA (45.7 ± 31.8 %), from 1.2 ± 0.1 % (*Micrococcus* sp.) to 103.6 ± 10.8 % (*Brevibacterium* sp.)

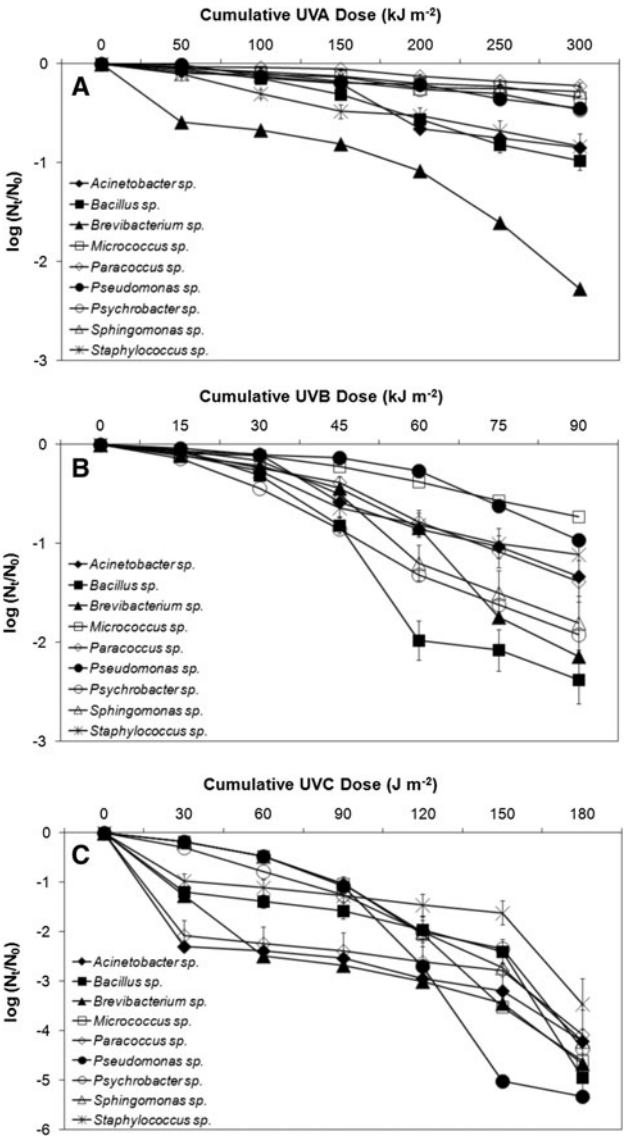

Fig. 1 UV sensitivity curves for the bacterial isolates under the different UV spectral regions. *Error bars* are standard deviation of the mean of three experiments. Where *error bars* are not displayed, they are smaller than the graph symbol. N_t number of CFU at the dose *t*. N_0 number of CFU at time 0

under UVB (36.1 ± 30.9 %), and from 6.3 ± 0.7 % (*Sphingomonas* sp.) to 68.4 ± 6.8 % (*Staphylococcus* sp.) under UVC (26.5 ± 21.8 %) (Fig. 3d).

Antioxidant enzyme activity

The effects of UVA on CAT activity (51.6 ± 48.3 %) (Fig. 3e) ranged from a 62.8 ± 6.9 % inhibition (*Acinetobacter* sp.) to a 99.5 ± 9.8 % stimulation (*Brevibacterium* sp.). The effects of UVB (8.3 ± 49.7 %) varied between a 89.1 ± 6.5 % reduction (*Micrococcus* sp.) and a 62.9 ± 6.6 % increase (*Brevibacterium* sp.), and UVC

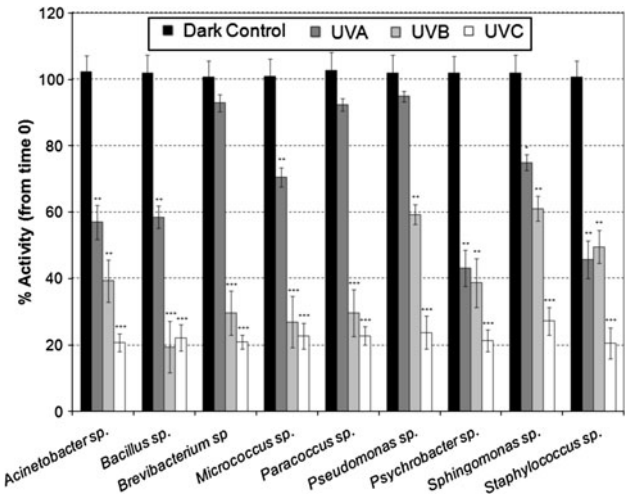

Fig. 2 Effects of exposure to the LD_{50} of different UV spectral regions on bacterial activity. LD_{50} values are shown in Table 1. Data are presented as group means ± standard deviations of the mean of three experiments. Absence of *error bars* indicates that standard deviations are too small to see on the scale used

effects (19.4 ± 25.1 %) ranged from an inhibition of 29.3 ± 3.1 % (*Bacillus* sp.) to a 48.8 ± 5.8 % increase (*Paracoccus* sp.) in CAT activity.

Irradiation with UVA and UVC caused an overall decrease in SOD activity (by an average of 28.5 ± 25.8 % and 24.5 ± 20.1 %, respectively), while UVB caused an average increase of 21.5 ± 19.3 %. The effects of UVA ranged from a 53.0 ± 5.7 % inhibition (*Bacillus* sp.) to a 30.4 ± 3.0 % stimulation (*Staphylococcus* sp.) of SOD activity. UVB either had no significant effect (*Staphylococcus* sp.) or enhanced SOD activity by as much as 59.1 ± 6.2 % (*Brevibacterium* sp.), and UVC effects ranged from an inhibition of 47.3 ± 4.9 % (*Bacillus* sp.) to a 18.0 ± 1.7 % increase (*Staphylococcus* sp.) (Fig. 3f).

Differences between wavelengths and determinants of inactivation

PCA was applied to the data set to identify the main determinants of the differences between the effects of the three UV spectral regions tested (Fig. 4). The different radiation regimes were clearly separated along PC 1, with UVA treated samples displaying the lowest PC 1 scores, mostly related to ROS (−0.495), TBARS (−0.496) and carbonyl levels (−0.231). UVC-treated samples displayed the highest PC 1 scores, related to activity (0.484) and DSB (0.363), while UVB-treated samples were located in between UVA- and UVC-treated samples.

Multiple linear stepwise regression analysis was used to assess the main determinants of bacterial inactivation, expressed as LD_{50}, following exposure to UV radiation of different spectral regions. Results are presented in Table 2. Under UVA, 34.2 % of the variability in LD_{50} could be accounted for by DSB and SOD activity. Under UVB, 57.7 % of the variability in LD_{50} could be explained by the combination of TBARS, DSB and activity. Under UVC, ROS and DSB levels accounted with statistical significance for 55.8 % of the variation in LD_{50} values.

Discussion

In this study, the cellular and biological effects of UVA, UVB and UVC were assessed for a set of isolates characterized by different UV sensitivities using biological and biochemical methods. Additionally, multivariate analyses (principal component analysis and regression analysis) were conducted to identify the determinants of cell inactivation under different UV spectral regions.

Interspecific variation in UV sensitivity

Responses of the different isolates tested to each UV spectral region varied greatly. Several factors can account for this variability. Specifically, the preferential molecular target (e.g., nucleic acids, proteins, lipids) of UV radiation may differ among strains. For example, it has been suggested that the extent of UV-induced DNA damage in Gram-positive bacteria is lower than that in Gram-negative bacteria because of a shielding effect by the cell wall (Jagger 1985). Another factor is that the relative contribution of different reactive oxygen species involved in eliciting UV-induced damage may differ among strains, which may then affect the extent of the damage. For instance, the presence of high concentrations of intracellular iron in *Shewanella oneidensis* MR-1 promotes ROS proliferation and the production of the highly toxic hydroxyl radicals via Fenton-type chemistry; accordingly, this strain is extremely susceptible to UV radiation (Qiu et al. 2005). Finally, the efficiency of the defense and repair strategies to cope with damage may also differ among bacteria (Arrieta et al. 2000; Matallana-Surget et al. 2009b; Santos et al. 2011).

In the present study, the set of isolates tested showed in general a much higher resistance to UVA radiation than that reported in similar studies using *Shewanella oneidensis* MR1(Qiu et al. 2004) and *Escherichia coli* (Ubomba-Jaswa et al. 2009). UVB LD_{50} values were, on average, up to 10 times higher than those observed in Antarctic marine bacteria (Hernandez et al. 2006) as well as in a set of marine bacterial isolates and the enteric bacteria *Salmonella typhimurium* CIP 60.62^{T} (Joux et al. 1999). On the other hand, the average UVC LD_{50} (26.6 J m^{-2}) determined in the present study was lower than that detected in

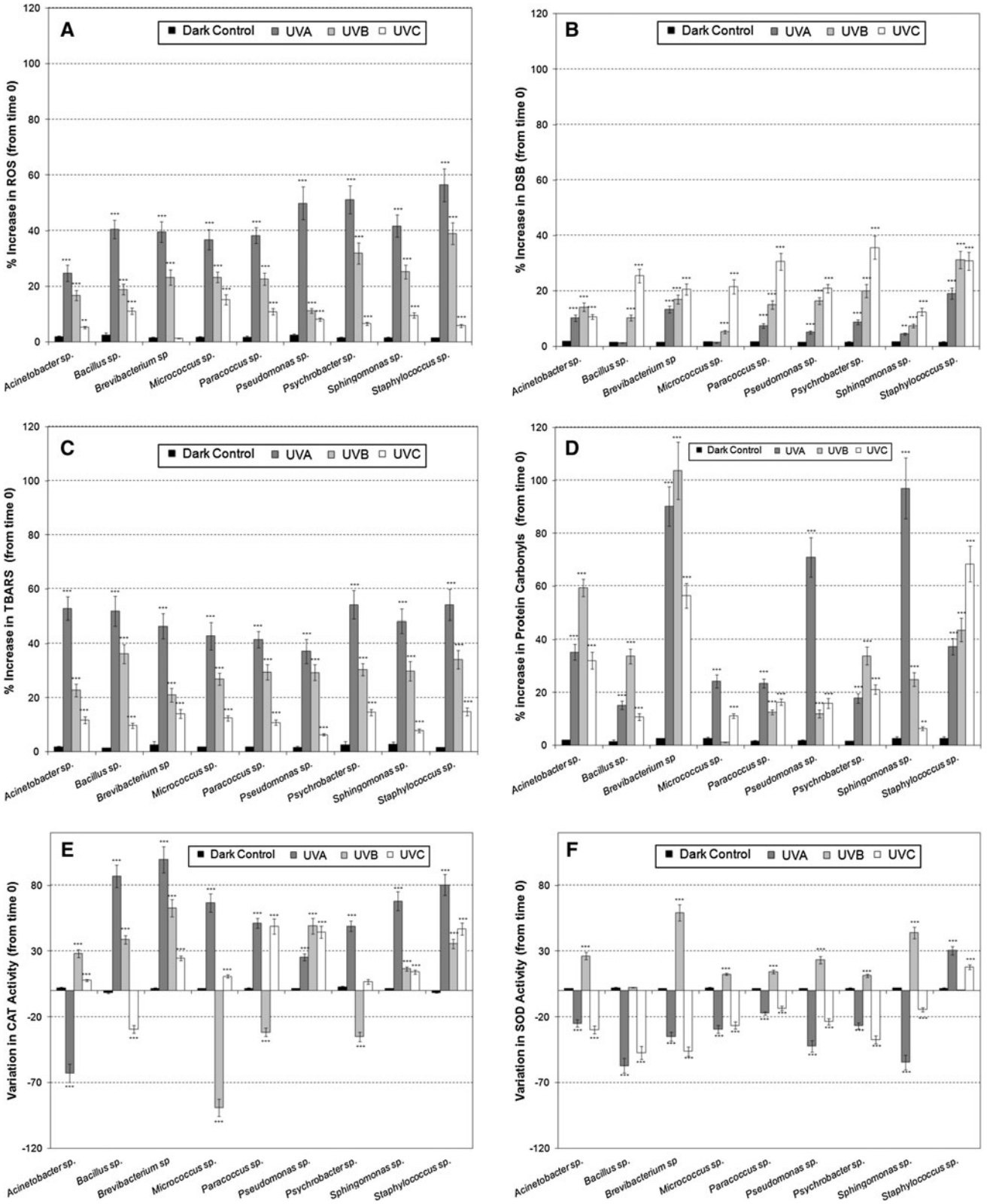

Fig. 3 Effects of exposure to the LD_{50} of different UV spectral regions on **a** intracellular ROS generation, **b** DSB, **c** TBARS levels, **d** protein carbonyl levels, **e** CAT and **f** SOD activity. LD_{50} values are shown in Table 1. Data are presented as group means ± standard deviations of the mean of three experiments. Absence of *error bars* indicates that standard deviations are too small to see on the scale used

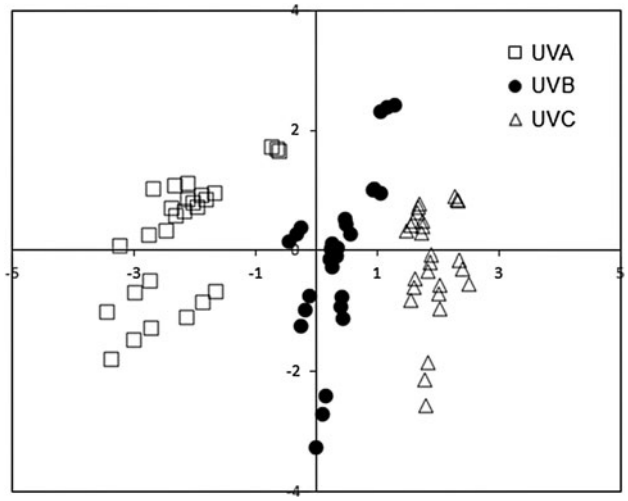

Fig. 4 Principal component analysis (PCA) score plot of data (activity, ROS levels, lipid oxidation, protein oxidation, DNA lesions, CAT and SOD activity) used to determine the parameters contributing the most for the separation of UVA, UVB and UVC treatments. A total of 27 data points (9 bacterial isolates × 3 replicates) was used for PCA analysis

Table 2 Multiple stepwise regression analysis used to determine the parameters that explained bacterial inactivation under the different UV spectral regions

	Adjusted R^2 of the model (p)	Predictor variable	β	p
UVA	0.342 (0.038)	DSB	−0.953	0.001
		SOD	0.557	0.038
UVB	0.577 (0.017)	TBARS	0.753	0.000
		DSB	−0.497	0.001
		Activity	−0.337	0.017
UVC	0.558 (0.003)	ROS	0.684	0.000
		DSB	−0.433	0.003

β standardized coefficient, p probability, R^2 coefficient of correlation
DSB DNA strand breaks, *SOD* superoxide dismutase, *ROS* reactive oxygen species, *TBARS* thiobarbituric acid reactive substances

Clavibacter michiganensis (UVC LD_{50} of approximately 75 J m^{-2}) (Jacobs and Sundin 2001) and even lower than the average minimal inhibitory UVC dose observed in a set of isolates retrieved from the peanut phyllosphere (Sundin and Jacobs 1999).

In this study, the Gram-positive, high G+C content *Actinobacteria Micrococcus* sp. showed the highest resistance to each UV spectral region. Highly UV-resistant *Micrococcus* strains have previously been isolated (Ordoñez et al. 2009). It has been suggested that Gram-positive bacteria are better adapted to UV stress because their cell walls screen out a considerable fraction of UV radiation (Jagger 1985). The genomic composition of microorganisms, particularly the G+C content and bipyrimidine nucleotide frequency, also affects the frequency and spectrum of DNA lesions formed during exposure to UV radiation (Matallana-Surget et al. 2008; Moeller et al. 2010). The high G+C content of *Actinobacteria* has been proposed to confer protective adaptation against UV radiation by minimizing the formation of cyclobutane dimers (Warnecke et al. 2005). However, the other *Actinobacteria* tested in this study (*Brevibacterium* sp.) was quite sensitive to UV radiation, which is in agreement with observations by Ordoñez et al. (2009), demonstrating that cell wall characteristics and G+C content are not the sole determinants of UV resistance. The presence of protective pigmentation (Shick and Dunlap 2002) and specialized DNA repair systems (Dodson et al. 1994) in *Micrococcus* sp., but not *Brevibacterium* sp., could contribute to the discrepancy in UV sensitivity of these *Actinobacteria*.

Gammaproteobacteria have been reported as the most UV-resistant group in several aquatic environments (Alonso-Sáez et al. 2006; Ordoñez et al. 2009; Santos et al. 2012b). In the present study, *Pseudomonas* sp. showed high levels of resistance to the different UV spectral regions, in agreement with previous studies (Ordoñez et al. 2009). UV-resistant *Acinetobacter* strains have also been isolated and their resistance associated with efficient DNA repair mechanisms (Fernández Zenoff et al. 2006; Hörtnagl et al. 2011) and high catalase activity (Di Capua et al. 2011). However, in this study, *Acinetobacter* sp. was found to be UV sensitive. UV-sensitive and UV-resistant *Acinetobacter* strains have been isolated even from the same environment (Ordoñez et al. 2009), suggesting that UV resistance is not a phylogenetic characteristic.

The alphaproteobacterium *Sphingomonas* sp. also showed high resistance to UV radiation, which is in accordance with previous reports of reduced accumulation of DNA lesions in *Sphingomonas* strains following UV exposure (Joux et al. 1999).

General trends in the effects of UV radiation

Despite the interspecies variability observed, some wavelength-dependent trends in the variation of biological and biochemical parameters studied were identified. Specifically, shorter UV wavelengths caused the greatest bacterial inactivation (denoted by lower UV doses being required to reduce bacterial numbers) and reduction in activity, while longer UVA wavelengths had more subtle effects in accordance with their indirect, ROS-mediated mode of action (Eisenstark 1998). Intracellular ROS generation, lipid oxidation (TBARS) and protein carbonylation, which are indicative of indirect UV effects, showed the strongest response to UVA irradiation. DSB formation resulting from direct interaction of UV with DNA was highest under UVC. The magnitude of UVB effects was generally

between those of UVA and UVC, supporting the suggestion that UVB-induced damage comprises elements from both the direct and indirect pathways of damage (Qiu et al. 2005). No wavelength-dependent variation was detected for CAT or SOD, suggesting that the type of ROS involved in eliciting the damage and the degree to which the interconnected mechanisms of ROS generation and removal by antioxidant enzymes act in each particular strain are extremely variable and ultimately shape the individual patterns of response of CAT and SOD to irradiation.

Principal component analysis applied to the entire data set clearly separated the effects of different irradiation treatments. The extent of the oxidative damage to lipids and proteins, as well as ROS levels, was found to be involved in differentiating UVA effects from the other UV regimes, suggesting that membrane lipids and proteins are major targets of UVA-induced oxidative modifications, in accordance with previous studies (Pizarro and Orce 1988; Bosshard et al. 2010b). The effects of UVC were more related to the extent of damage to DNA (assessed using DSB as a proxy), which is in agreement with the mutagenic nature of UVC wavelengths (Friedberg et al. 1995), as well as their inhibitory effects on activity. The PCA bidimensional plot showed that UVB-treated samples were positioned between UVA and UVC treatments, supporting the intermediate nature of the effects of UVB when compared with those of UVA and UVC (Qiu et al. 2005).

Determinants of bacterial inactivation

Multiple linear stepwise regression analysis was used to assess the main determinants of bacterial inactivation following exposure to different UV spectral regions. The amount of DSB emerged as the major determinant of inactivation upon exposure to UVA. UVA-induced damage has traditionally been attributed to photodynamic reactions mediated by cellular chromophores since DNA does not strongly absorb light in the UVA range (Cadet et al. 2005). More recently, investigations conducted on eukaryotes have highlighted the high mutagenic potential of UVA (Rünger et al. 2012) and the role of cyclobutane pyrimidine dimers, rather than oxidative lesions, in UVA-induced damage (Ikehata et al. 2008). The finding that UVA induced the lowest reduction in CFU among the investigated spectral regions demonstrates that bacteria are able to minimize UVA-induced DNA lesions. This may involve light-dependent repair mechanisms mediated by photolyase activated by UVA radiation itself, as well as light-independent repair (Mitchell and Karentz 1993). Additionally, most UVA sensitive strains displayed significantly higher levels of DSB and TBARS (ANOVA, $p < 0.05$). Taken together, these results suggest that the extent of oxidative damage to biomolecules and counteracting protective mechanisms underlies the variability in UVA susceptibility among different bacteria but that the accumulation of DNA damage ultimately leads to cell death.

Under UVB, oxidative damage to lipids (TBARS), accumulation of DNA damage and loss of metabolic activity were the main determinants of inactivation. These results indicate that, in addition to DNA damage, changes in the integrity of membrane structure and functionality during UVB exposure play an important role in bacterial inactivation. Such changes may compromise the ability of the cell to generate energy necessary to sustain its activity and elicit repair strategies following irradiation (Bosshard et al. 2010a). Most UVB sensitive strains displayed significantly higher levels of protein carbonyls than resistant ones (ANOVA, $p < 0.05$), which also identifies protein oxidation as an important determinant of bacterial susceptibility to UVB radiation. UVB was the only irradiation regime to enhance SOD activity levels in all bacteria when compared with non-irradiated controls, indicating that SOD may play an important protective role against UVB. The superoxide radical is able to directly cause oxidative damage to the bases of DNA (Misiaszek et al. 2004). Additionally, superoxide can attack Fe–S clusters of enzymes, rendering them inactive. The released ferrous iron can, in turn, react with H_2O_2, resulting in the formation of the highly toxic hydroxyl radical which is able to attack virtually any biomolecule (Imlay 2006).

Regression analysis revealed that ROS levels together with DNA damage were the best predictors of cell inactivation under UVC. However, the extent of DNA damage did not differ significantly between resistant and sensitive strains, which is in accordance with previous observations (De La Vega et al. 2005). On the other hand, most UVC-sensitive strains displayed significantly higher TBARS and carbonyls levels than resistant ones (ANOVA, $p < 0.05$), which suggests that the extent of oxidative damage to lipids and proteins interferes with vital biological functions and is therefore an important component of UVC-induced inactivation (Krisko and Radman 2010; Schenk et al. 2011). Unexpectedly, ROS levels were significantly higher in resistant strains (ANOVA, $p < 0.05$); however, it is unclear whether these findings have any biological significance. Accordingly, additional studies to investigate whether ROS generation is involved in eliciting specific defense strategies in response to UVC exposure, as in cyanobacteria (Dillon et al. 2002) and plants (Murphy and Huerta 1990), are warranted.

New insights into the mechanisms of UV-induced damage in bacteria

The present work aimed to dissect the wavelength dependence of the damage induced by UV radiation. In order to

do so, a combination of a multitude of UV-sensitivity tests and their statistical analysis was applied. Using this combined innovative approach, new clues regarding the targets of UV radiation of different spectral regions across a range of bacteria with different UV susceptibilities emerged. In particular, the involvement of DNA damage in eliciting bacterial inactivation upon UVA exposure was observed, which is in accordance with work reporting the induction of the SOS response in UVA-irradiated bacteria (Qiu et al. 2005; Berney et al. 2006a). Oxidative damage to lipids was found to be determinant for bacterial inactivation during UVB exposure. Such observation is in agreement with recent reports of enhanced expression of the glyoxalase protein and alkyl hydroperoxide reductase AhpC, involved in the detoxification of lipid peroxidation by-products, following UVB exposure of *Photobacterium angustum* S14 (Matallana-Surget et al. 2012). Finally, oxidative stress was also found to be crucial for cell inactivation under UVC, supporting evidence accumulating in recent years (Gomes et al. 2005; Krisko and Radman 2010; Schenk et al. 2011).

Most investigations on the effects of UV radiation on bacteria have been conducted on a small number of genetically well-characterized microorganisms that are not always representative of natural environmental communities. By using bacterial strains originating from a photo-stressed microbial community (Santos et al. 2011), the information gained from the isolates used in the present work could provide clues to understand how natural microbial assemblages might react to global changes, particularly changes in environmentally relevant UV radiation.

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