

Genomic bipyrimidine nucleotide frequency and microbial reactions to germicidal UV radiation

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Abstract The role of the genomic bipyrimidine nucleotide frequency in pyrimidine dimer formation caused by germicidal UV radiation was studied in three microbial reference organisms (*Escherichia coli* K12, *Deinococcus radiodurans* R1, spores and cells of *Bacillus subtilis* 168). The sensitive HPLC tandem mass spectrometry assay was used to identify and quantify the different bipyrimidine photoproducts induced in the DNA of microorganisms by germicidal UV radiation. The yields of photoproducts per applied fluence were very similar among vegetative cells but twofold reduced in spores. This similarity in DNA photoreactivity greatly contrasted with the 11-fold range determined in the fluence causing a decimal reduction of survival. It was also found that the spectrum of UV-induced bipyrimidine lesions was species-specific and the

formation rates of bi-thymine and bi-cytosine photoproducts correlated with the genomic frequencies of thymine and cytosine dinucleotides in the bacterial model systems.

Keywords *Bacillus* spores · *Deinococcus radiodurans* · *Escherichia coli* · Germicidal UV radiation · DNA photolesions · Bipyrimidine photoproducts

Introduction

Ultraviolet (UV) disinfection is an environmentally safe method for inactivating microorganisms, especially as an alternative to chemical sterilization approaches where further reactions resulting in potentially noxious chemical complexes can occur (Chang et al. 1985; Blatchley et al. 2000; Eiseheid and Linden 2007; Eiseheid et al. 2009). Application of short wavelength UV radiation (UV-C) produced by low-pressure mercury lamps emitting mostly 254-nm (germicidal UV) is a standard practice for efficient and cost-effective disinfection of surfaces (Stannard et al. 1985), of air inside rooms (Nakamura 1987), and of water (Lazarova and Savoys 2004). UV sterilization is unmatched in its efficiency, simplicity, and dependability when applied as a microorganism disinfectant. The effectiveness of UV-C radiation as disinfectant arises primarily from the fact that DNA molecules absorb UV photons between 200 and 300 nm, with peak absorption around 260 nm, very close to the 254-nm radiation emitted by low-pressure mercury lamps (Douki and Cadet 2001; Sinha and Häder 2002; Douki et al. 2003; Cadet et al. 2005). Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) are the major photo-induced DNA lesions that are formed at DNA bipyrimidine sites in irradiated microorganisms, while strand breaks and

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DNA–protein-crosslinks are only produced in minor quantities (Harm 1980; Cadet et al. 2005 and references therein). In hydrated cells, both CPDs and 6-4PPs can be produced at any of the four bipyrimidine dinucleotides, although in different yields (Douki and Cadet 2001, 2003; Cadet et al. 2005). In dry DNA and DNA of bacterial endospores, UV-C induces predominantly 5,6-dihydro-5(α -thymine)thymine, the so-called spore photoproduct (SP) (Donnellan and Setlow 1965; Varghese 1970).

UV photoproducts within DNA are generally measured by two alternative approaches: (1) immunoassays using antibodies that specifically recognize CPDs and 6-4PPs as classes of bipyrimidine photoproducts (BPPs) and (2) chromatographic assays that simultaneously monitor a variety of photo-induced base lesions in DNA isolated from irradiated microorganisms (Cadet et al. 2005; Douki et al. 2005a, b; Pogoda de la Vega et al. 2005; Moeller et al. 2007a, b). The sensitive HPLC–MS/MS technique operating in the electrospray ionization (ESI) detection mode belongs to this last class. It has not only enabled the determination of the total amount of BPPs but also the identification and quantification of each individual type of DNA photoproduct formed in UV-irradiated microorganisms (Douki and Cadet 2001, 2003; Cadet et al. 2005; Matallana-Surget et al. 2008, 2009).

Recently, Matallana-Surget et al. (2008) made an interesting observation on the effect of the GC content of DNA on the distribution of UV-B induced BPPs. They reported that both the frequency and spectrum of BPPs was altered as a function of the genomic GC content. Broad spectrum UV-B radiation was applied to three different microorganisms, i.e. two marine bacteria, namely *Sphingopyxis alaskensis* RB2256 and *Vibrio angustum* S14, and the food-poisoning pathogen *Salmonella typhimurium* LT2. In addition to this set of microorganisms, they also irradiated commercially available DNA from *Clostridium perfringens*, calf thymus and *Micrococcus luteus*.

However, the overwhelming majority of published papers on the effects of UV on bacteria have been conducted (1) using germicidal monochromatic 254 nm UV radiation and (2) on genetically well-characterized microorganisms (Coohill and Sagripanti 2008, 2009 and references therein). Therefore, we followed up the interesting preliminary observation by Matallana-Surget et al. (2008) by using three of the well-characterized and most frequently tested microbial model systems in studying the microbial response to germicidal as well as environmentally relevant UV radiation, i.e. *Escherichia coli* K12, *Deinococcus radiodurans* R1, cells and endospores (spores) of *Bacillus subtilis* 168 (Nicholson and Galeano 2003; Pogoda de la Vega et al. 2005; Moeller et al. 2007a, c, 2009; Bauermeister et al. 2009). These bacteria have often been used as surrogates for pathogenic and food-poisoning

bacteria such as *B. anthracis* and *B. cereus* (Nicholson and Galeano 2003; Weber et al. 2003) or *Escherichia coli* O157:H7 (Oguma et al. 2001), as well as UV biosimetry surrogates for drinking water contaminants (Chang et al. 1985; Marshall et al. 2003; Deller et al. 2006).

In this short communication, we report on the impact of the genomic frequencies of bipyrimidine nucleotide combination on the formation of DNA photoproducts induced by germicidal 254-nm UV-C radiation. The UV-C induced BPPs were quantified by an HPLC–MS/MS approach. Emphasis was placed in this study on the quantitative characterization and discrimination of UV-C induced DNA base photolesions. Besides survival data, we present the spectrum of induced BPPs encountered within the DNA of the irradiated cells and discuss the role of genomic pyrimidine dinucleotide frequencies on the specificity of 254 nm UV-induced DNA photoproducts to strengthen the findings on the impact of GC content on the induction and distribution of BPP earlier reported by Matallana-Surget et al. (2008) after exposure to environmentally relevant polychromatic UV-B radiation.

Materials and methods

Bacterial strains, media and culturing conditions

B. subtilis 168 (DSM 402), *D. radiodurans* R1 (DSM 20539), *E. coli* K12 (DSM 498) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) GmbH, Braunschweig, Germany. Cells of *B. subtilis* and *E. coli* were cultivated under vigorous aeration at 37°C in nutrient broth (NB) medium (Difco, Detroit, MI) and *D. radiodurans* in tryptone-glucose-yeast (TGY) broth at 30°C as described previously (Oguma et al. 2001; Pogoda de la Vega et al. 2005; Moeller et al. 2007b, 2009). All vegetative cells were grown in liquid culture to mid-exponential growth phase, as monitored by OD measurement at 600 nm of 0.7 ± 0.1 . For vegetative cells of *B. subtilis*, only suspensions with a spore fraction less than 0.01% were used, as determined by heat shock at 80°C for 15 min. Spores of *B. subtilis* were obtained by cultivation under vigorous aeration in double-strength liquid Schaeffer Sporulation Medium (Nicholson and Setlow 1990; Moeller et al. 2009), purified and stored as described previously (Moeller et al. 2007a, c, 2009).

UV-C irradiation

Cell suspensions in 25 ml of phosphate-buffered saline and spores in aqueous suspension at a concentration of 5×10^7 /ml were exposed to 254 nm UV radiation emitted from a low-pressure mercury lamp (Model NN 8/15,

Heraeus, Berlin, Germany). The spectral irradiance was determined spectrophotometrically (Bentham 150 double monochromator) and the fluence rate was 75.8 W/m^2 as measured with a UV-X radiometer (UVP Ultra-Violet Products, Cambridge, UK) (Moeller et al. 2005; Pogoda de la Vega et al. 2005). Each irradiation was carried out at 4°C while the suspensions were continuously stirred to ensure homogeneous exposure (Pogoda de la Vega et al. 2005; Moeller et al. 2007a, c, 2009). To avoid photoreactivation by visible light, UV irradiation was performed in a black-painted photo-laboratory under yellow light at the German Aerospace Center, Institute for Aerospace Medicine, Cologne, Germany.

Quantification of BPPs

For DNA photoproduct analyses, chromosomal DNA was extracted from irradiated samples by using the Wizard Genomic DNA Purification Kit (Promega GmbH, Mannheim, Germany) procedure for genomic DNA isolation from bacteria (Pogoda de la Vega et al. 2005; Moeller et al. 2007a, c), whereas spores were chemically deoated prior DNA isolation as previously described by Sargent (1980). The extracted DNA was then enzymatically digested by nuclease P1, phosphodiesterases I and II and alkaline phosphatase, and dimeric photoproducts were released as modified dinucleoside monophosphates while normal bases were obtained as 2'-deoxyribonucleosides (Douki et al. 2005a, b; Pogoda de la Vega et al. 2005; Moeller et al. 2007a, c). BPPs that include CPDs [TT (thymine-thymine), TC (thymine-cytosine), CT (cytosine-thymine) and CC (cytosine-cytosine)] along with the corresponding 6-4PPs and related Dewar valence isomers (DEWs) as well as SP, all deriving from intrastrand photoreactions at bipyrimidine sites, were quantified by HPLC–MS/MS (Douki and Cadet 2001, 2003; Cadet et al. 2005).

Survival assay

Cell survival of the irradiated microorganisms was determined by assessing their ability to form macroscopic visible colonies (i.e. colony forming units, CFU) on nutrient agar plates after incubation under optimal growth conditions as described previously (Pogoda de la Vega et al. 2005; Moeller et al. 2007a, c).

Numerical and statistical analysis

Best-fit curves were used to calculate characteristic inactivation values for statistical comparison (Pogoda de la Vega et al. 2005; Moeller et al. 2007a, c). To determine the survival curve parameters, the following relationship was used: $\ln(N/N_0) = -k_i \times F + n$, with $N = \text{CFU/ml}$ after

irradiation, $N_0 = \text{CFU/ml}$ without irradiation, $k_i = \text{inactivation constant (m}^2/\text{J)}$, $F = \text{UV fluence (J/m}^2)$ and $n = \text{extrapolation number, i.e. the intercept with the ordinate of the extrapolated semi-logarithmic straight line}$. The constants k_i and n were determined by linear regression. Photoproduct induction curves were obtained by plotting the total number of BPPs per 10^4 bases versus fluence. The efficiency of photoproduct induction was determined from $\text{PP} = k_{\text{PP}} \times F$, with $\text{PP} = \text{BPPs}/10^4$ bases, $k_{\text{PP}} = \text{photo-product induction constant (BPPs}/10^4 \text{ bases per J/m}^2)$. k_{PP} was determined by linear regression of the induction curves (Pogoda de la Vega et al. 2005; Moeller et al. 2007a, c). The response of microorganisms to UV radiation was described by reporting the F_{10} -value, which is defined as the UV fluence which reduces cell viability by a factor of 10 (Nicholson and Galeano 2003; Moeller et al. 2009). To measure intrinsic differences in the UV-C induced inactivation, all strains were treated in parallel under identical conditions of growth, irradiation, and survival testing. All experiments were repeated four times. The data shown are mean values \pm standard deviation. The significance of the differences in the survival rates, BPPs induction and distribution were determined by analysis of variance (ANOVA), using *Analyse-it* software (*Analyse-It* Software, Ltd., Leeds, UK). Values were evaluated in multigroup pairwise combinations, and differences with P values of ≤ 0.05 were considered statistically significant (Pogoda de la Vega et al. 2005; Moeller et al. 2007a, b, 2009).

Results and discussion

Inactivation rates by UV-C radiation

Figure 1 shows the characteristic survival curves of the different test systems as semi-logarithmic plots of the survival fraction versus UV fluence. Strictly exponential inactivation was obtained for *E. coli* and *B. subtilis*, whereas shoulder curves were obtained for *D. radiodurans* (Fig. 1a). F_{10} -values were derived from these fluence-effect curves of inactivation (Table 1). Examination of the F_{10} -values showed that *E. coli* was the most UV-sensitive microorganism ($F_{10} = 23 \pm 5 \text{ J/m}^2$). The UV-resistance of the test systems increased in the following order: *E. coli*–*B. subtilis* cells–*B. subtilis* spores–*D. radiodurans* (Table 1). *D. radiodurans* ($F_{10} = 603 \pm 59 \text{ J/m}^2$) was approximately 3 times more resistant to UV-C radiation than *B. subtilis* spores ($F_{10} = 221 \pm 38 \text{ J/m}^2$), while vegetative cells of *B. subtilis* ($F_{10} = 53 \pm 8 \text{ J/m}^2$) were fourfold more sensitive than their spore counterparts (Table 1). These F_{10} values of the UV-irradiated microorganisms were in good agreement with published data by various authors (Nicholson et al. 2000; Oguma et al. 2001;

Fig. 1 Fluence-survival (open symbols; **a**) and induction of BPPs (filled symbols; **b**) of *D. radiodurans* (circles), *E. coli* (squares), *B. subtilis* cells (triangles down) and spores (triangles up) after exposure to UV-C radiation. Data are averages \pm standard deviation ($n = 4$). Otherwise error bars for survival data not shown were smaller than the symbol

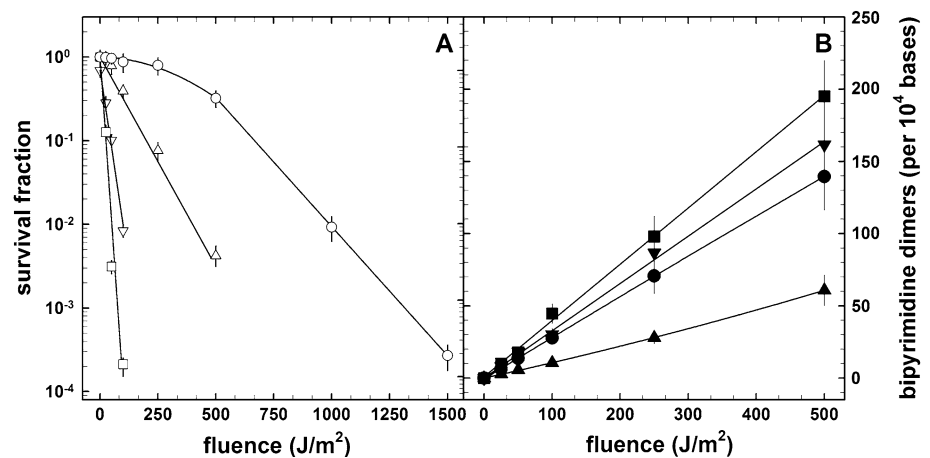


Table 1 Parameter of the (254 nm) UV resistance and BPPs formation of the tested bacterial species

Strain	F_{10}	k_i	Ratio k_i^a	k_{pp}	Ratio k_{pp}^b
<i>B. subtilis</i> (cells)	$53.4 \pm 8.1^*$	$(2.7 \pm 0.3) \times 10^{-2*}$	$2.9 \pm 0.4^*$	$(3.2 \pm 0.4) \times 10^{-1}$	1.2 ± 0.2
<i>B. subtilis</i> (spores)	$220.8 \pm 38.0^*$	$(1.4 \pm 0.3) \times 10^{-2*}$	$1.9 \pm 0.2^*$	$(1.2 \pm 0.2) \times 10^{-1*}$	$0.4 \pm 0.1^*$
<i>D. radiodurans</i>	603.2 ± 59.4	$(7.6 \pm 0.9) \times 10^{-3}$	1	$(2.7 \pm 0.3) \times 10^{-1}$	1
<i>E. coli</i>	$23.1 \pm 5.2^*$	$(8.7 \pm 1.1) \times 10^{-2*}$	$11.5 \pm 1.8^*$	$(4.0 \pm 0.3) \times 10^{-1}$	1.5 ± 0.3

Data reported as averages \pm standard deviations ($n = 4$). Asterisks indicate F_{10} values and k_{pp} values that were significantly different (P values of ≤ 0.05) than the respective values for *D. radiodurans*

^a Ratio of k_i (*D. radiodurans*) to the k_i of the other tested bacterial systems (i.e. *E. coli*, spores and cells of *B. subtilis*)

^b Ratio of k_{pp} (*D. radiodurans*) to the k_{pp} of the other tested bacterial systems (i.e. *E. coli*, spores and cells of *B. subtilis*)

Nicholson and Galeano 2003; Pogoda de la Vega et al. 2005; Moeller et al. 2007a, c).

Induction of BPPs

While survival determined from CFU is an indicator of the responses of whole cells to UV-C radiation, measurement of BPPs provides data on the molecular effects of UV-C radiation on DNA in the irradiated microorganisms. Since inactivation of microorganisms by UV-C radiation is effected through the formation of DNA lesions, a comparison of CFU and BPPs data may help to delineate UV biological consequences on the whole cell from its effects on the DNA (Setlow and Carrier 1966; Harm 1980; Sinha and Häder 2002; Cadet et al. 2005). For all three tested bacterial model systems, a linear increase in the total amount of BPPs per 10^4 bases with the applied fluence was found (Fig. 1b). The formation of BPPs, as determined from their PP induction constant (k_{pp}), is ranged according to the microorganisms in the following increasing order: *B. subtilis* spores < *D. radiodurans* < *B. subtilis* cells < *E. coli*. However, the differences in k_{pp} between vegetative cells of *B. subtilis*, *D. radiodurans* and *E. coli* were statistically not significant, whereas the k_{pp} of the *B. subtilis* spores was twofold lower than those of the vegetative cells,

as shown in the ratio of the total k_{pp} values (Table 1). When comparing the different test systems with regard to their rates of UV-survival and BPPs formation, we found dramatic differences (up to 11.5 times) in the survival, but only weak differences in the induction of BPPs (Table 1).

Spectrum of the UV-C induced BPPs

CPDs were found to be the major lesions in all UV-C treated vegetative cells; however, significant differences in the fraction of the major and minor induced DNA photo-products were detected between the different cellular systems (Table 2). In the DNA of UV-C irradiated *D. radiodurans*, the CPD TC was the major lesion and its fraction ($47.2 \pm 6.3\%$) amounted to almost half of all BPPs, while CPD TT ($25.4 \pm 5.1\%$), CPD CT ($14.6 \pm 3.4\%$), CPD CC ($7.3 \pm 1.7\%$) and 6-4 TC ($6.2 \pm 1.9\%$) were formed in moderate yields. The major UV-C induced BPPs in *E. coli* were CPD TC ($38.3 \pm 6.2\%$) and CPD TT ($34.5 \pm 3.1\%$) in almost equal quantities, while 6-4 TC ($10.8 \pm 3.8\%$) and CPD CT ($10.2 \pm 2.0\%$) were formed at a moderate rate. The major photolesions in vegetative *B. subtilis* cells were CPD TT ($53.1 \pm 3.2\%$) and CPD TC ($21.1 \pm 4.3\%$), while CPD CC ($3.2 \pm 0.7\%$) and 6-4 TC ($15.7 \pm 3.8\%$) were formed

Table 2 Individual and total photoproduct induction constants (k_{PP}) of the tested bacterial species

k_{PP} (m^2/J)	<i>B. subtilis</i> (cells)	<i>B. subtilis</i> (spores)	<i>D. radiodurans</i>	<i>E. coli</i>
6-4 ^a TC	$(5.3 \pm 0.7) \times 10^{-2*}$	$(4.6 \pm 0.3) \times 10^{-3*}$	$(1.9 \pm 0.3) \times 10^{-2}$	$(4.6 \pm 0.8) \times 10^{-2*}$
6-4 ^a TT	$(4.8 \pm 0.4) \times 10^{-3*}$	$(4.7 \pm 0.6) \times 10^{-4*}$	$(1.3 \pm 0.2) \times 10^{-3}$	$(3.5 \pm 0.6) \times 10^{-3*}$
CPD ^b CC	$(1.0 \pm 0.1) \times 10^{-2*}$	$(3.3 \pm 0.6) \times 10^{-4*}$	$(1.9 \pm 0.3) \times 10^{-2}$	$(1.3 \pm 0.2) \times 10^{-2*}$
CPD ^b CT	$(1.7 \pm 0.3) \times 10^{-2*}$	$(7.2 \pm 0.9) \times 10^{-4*}$	$(3.9 \pm 0.5) \times 10^{-2}$	$(4.3 \pm 0.6) \times 10^{-2}$
CPD ^b TC	$(7.1 \pm 1.2) \times 10^{-2*}$	$(3.2 \pm 0.4) \times 10^{-3*}$	$(1.3 \pm 0.2) \times 10^{-1}$	$(1.5 \pm 0.2) \times 10^{-1}$
CPD ^b TT	$(1.8 \pm 0.2) \times 10^{-1*}$	$(3.1 \pm 0.5) \times 10^{-3*}$	$(6.8 \pm 0.5) \times 10^{-2}$	$(1.3 \pm 0.1) \times 10^{-1*}$
DEW ^c TC	$(1.3 \pm 0.5) \times 10^{-3*}$	$(2.2 \pm 0.6) \times 10^{-4}$	$(3.8 \pm 0.9) \times 10^{-4}$	$(1.8 \pm 0.6) \times 10^{-3*}$
DEW ^c TT	$(8.3 \pm 0.5) \times 10^{-4*}$	$(1.5 \pm 0.2) \times 10^{-4*}$	$(3.6 \pm 0.2) \times 10^{-4}$	$(6.1 \pm 0.7) \times 10^{-4*}$
SP ^d	n.d.	$(1.1 \pm 0.2) \times 10^{-1*}$	n.d.	n.d.
Total	$(3.2 \pm 0.4) \times 10^{-1}$	$(1.2 \pm 0.2) \times 10^{-1*}$	$(2.7 \pm 0.3) \times 10^{-1}$	$(4.0 \pm 0.3) \times 10^{-1*}$

n.d. not determinable

Data reported as averages \pm standard deviations ($n = 4$). Asterisks indicate PP induction constant values that were significantly different (P values of ≤ 0.05) than the respective values for *D. radiodurans*

in moderate quantities. CPD CT and 6-4 TT add up to 2% of the total BPPs and slight traces of DEW TC ($0.4 \pm 0.1\%$), corresponding to approximately 10 DEW TC photolesion/ 10^9 bases per J/m^2 , were detected (Table 2). SP was only found in the UV-C treated *B. subtilis* spores, representing the major photoproduct ($93.7 \pm 5.6\%$) that is formed at the expense of minor CPD TC ($2.7 \pm 0.8\%$), CPD TT ($2.4 \pm 0.7\%$), 6-4 TC ($3.8 \pm 1.2\%$), and 6-4 TT ($0.4 \pm 0.1\%$). These results are in good agreement with previous observations (Douki et al. 2005a, b; Moeller et al. 2007a, b). It is clearly seen that the spectrum of BPPs formed in the DNA of UV-C irradiated dormant spores is significantly different from that of vegetative *B. subtilis* cells (Table 2). This unique photochemistry of bacterial endospores is mainly based on the A-DNA helix structure that is predominant during sporulation (Nicholson et al. 2000, 2005; Setlow 2006 and references therein). In all measured DNA BPPs, only minor levels of Dewar valence isomers (DEW TT and TC) were detected.

Relationship between decimal reduction of survival (F_{10} -values) and induced BPPs

The data in Fig. 2 shows the number of UV-C induced BPPs that were required to reduce the survival of the different test systems by one order of magnitude, i.e. at the respective F_{10} -value (Fig. 2). To obtain the same level of inactivation, different amounts of BPPs were required by the test systems. Approximately $53,720 \pm 5,290$ BPPs were necessary for the inactivation of 90% of the initial *D. radiodurans* cells, whereas spores of *B. subtilis* required about 5 times fewer BPPs ($10,742 \pm 1,852$) for the same level of reduction, and cells of *B. subtilis* and *E. coli* up to 12 times fewer BPPs ($7,155 \pm 1,085$ and $4,328 \pm 974$, respectively) for reaching their respective F_{10} values.

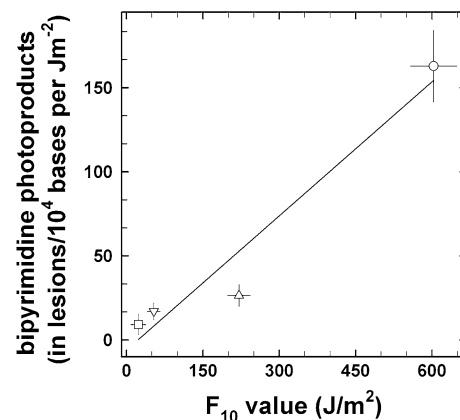


Fig. 2 Relationship between the fluence causing decimal reduction of survival (F_{10} value) and the number of BPPs (in BPPs/ 10^4 bases per J/m^2) at the individual F_{10} value in *D. radiodurans* (circles), *E. coli* (squares), *B. subtilis* cells (triangles down) and spores (triangles up). The regression on a straight line was determined by the least-squares method for four independent series of experiments. Coefficient of determination (r^2) was 0.942 for four data sets. Data are reported as averages and standard deviations ($n = 4$)

When assuming that UV radiation damages randomly the DNA in UV-exposed cell(s) and each microbial system, i.e. single cell (*E. coli* and *B. subtilis*) or spore (*B. subtilis*) (Nicholson et al. 2005), contains at least one genomic unit, one can calculate the mean frequency of UV-induced BPPs in the DNA for the F_{10} -value of each microorganism. It follows that on the average one BPP was formed every 392 ± 67 bp in *B. subtilis* spores, every 589 ± 88 bp in *B. subtilis* cells and every $1,072 \pm 219$ bp in *E. coli* cells for UV-C fluences corresponding to 10% survival. Genomic information on the number of chromosomes and plasmids of the investigated microbial model systems was used according to the TIGR database (<http://cmr.tigr.org>) (Peterson et al. 2001). In short, *B. subtilis* 168 has a genome size of 4,214,814 bp, *D. radiodurans* R1 with two

chromosomes (chromosome I: 2,648,638 bp and chromosome II: 412,348 bp), a megaplasmid (megaplasmid MP1: 177,466 bp), a small plasmid (plasmid CP1: 45,704 bp), creating a genome size of total 3,284,156 bp for *D. radiodurans* R1, and *E. coli* K12 has a single chromosome with 4,639,221 bp. However, the situation is somewhat different for UV-C irradiated *D. radiodurans* cells because >95% of cells were present as tetrads, and each cell of *D. radiodurans* is estimated to contain approximately four chromosomes (Hanson 1978; Tirgari and Moseley 1980). In that case, at the F_{10} value, each single coccus of a *D. radiodurans* tetrad showed on average one BPP every 245 ± 24 bp. Comparing these values with the individual genome size, at 90% inactivation, approximately $1.6 \pm 0.2\%$ of the total bp of *D. radiodurans* were damaged by irradiation, whereas an amount of $0.09 \pm 0.02\%$ base dimerization of the total bp of *E. coli* led to decimal reduction caused by UV exposure. When comparing cells and spores of *B. subtilis* at their F_{10} value, their genomes are $0.17 \pm 0.03\%$ and $0.26 \pm 0.04\%$, respectively dimerized by UV-C when their survival is reduced by one order of magnitude. Clearly, these results we obtained in this study show a linear relationship between the fluence causing a decimal reduction of survival (F_{10} value) and the total number of BPPs induced in the DNA of the irradiated microorganisms (Fig. 2). Concluding, the formation and distribution of BPPs in microbial DNA is primarily responsible for UV-C inactivation.

Correlations between genomic GC, C and T content and the BPPs induction

In our study, we used the frequency of pyrimidine dinucleotides in the published genomic sequences of the three test microorganisms, available at the NCBI “Complete Microbial Genomes” database (<http://www.ncbi.nih.gov/genomes/lproks.cgi>), to determine to dependency of the individual genomic bipyrimidine frequencies on the yield of BPPs upon UV-C irradiation. In Table 3, the frequencies of bipyrimidine dinucleotides (TT, CC, TC, CT) of the three bacterial species to their corresponding GC are shown. When analyzing the genomic bipyrimidine frequencies of the three bacterial species, it was observed that *D. radiodurans* has a relatively high CC (8.92%) and a low TT (4.02%) dinucleotide frequency; in contrast, the

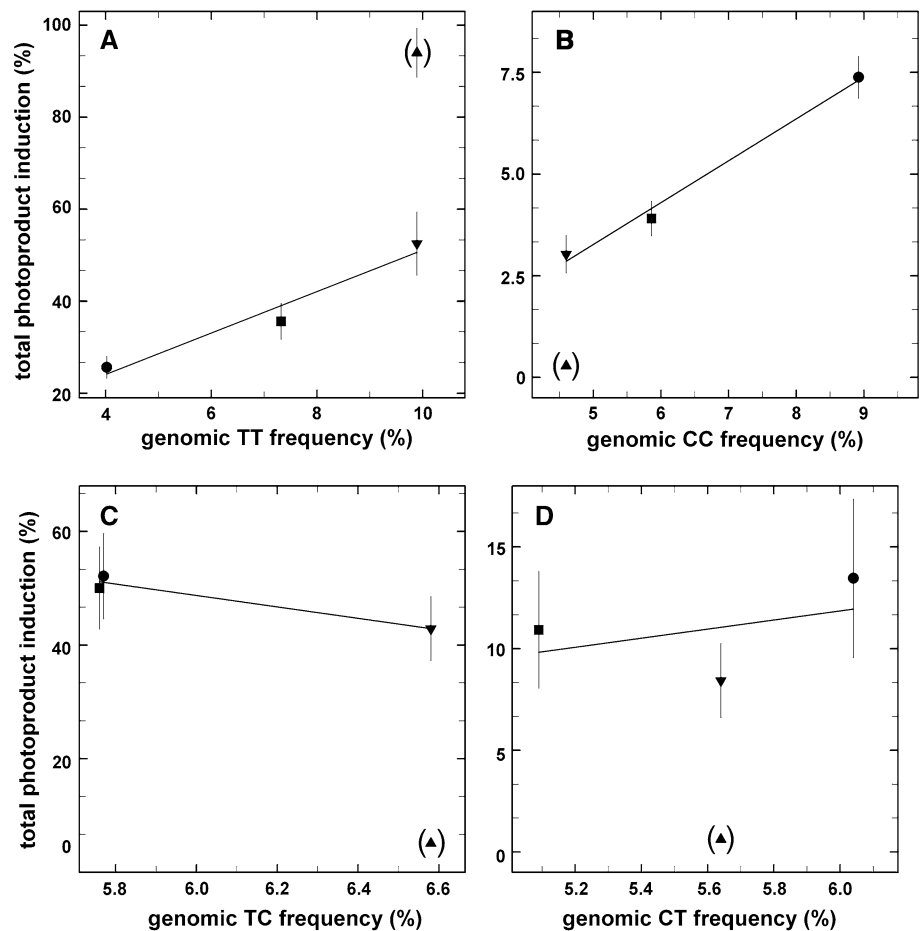
genome of *B. subtilis* contains a relatively high TT (9.89%) and low CC (4.60%) frequency (Table 3). *E. coli* is situated between *B. subtilis* and *D. radiodurans* with a genomic CC and TT dinucleotide frequency of 5.86 and 7.32%, respectively (Table 3). Only minor differences were observed in the TC (5.76–6.58%) and CT (5.09–6.04%) dinucleotide frequency ranges between the three investigated species (Table 3). In Fig. 3, the level of induced BPPs as a function of the genomic bipyrimidine nucleotide frequency of the three bacterial species is depicted, for vegetative cells of all bacterial species and in brackets for *B. subtilis* spores, used here in this work as one of the most used biosimetric system for measuring microbial response to UV radiation (Munakata 1981; Munakata et al. 2000; Marshall et al. 2003). It should be kept in mind that even when spores of *B. subtilis* were often used as surrogate for other spore-forming *Bacillus* species, e.g. *B. cereus* and *B. anthracis*, there are several differences in the strain-specific spore resistance (Sagripanti et al. 2007; Carrera et al. 2008). However, similar response to UV-C resistance of spores of various *Bacillus* species, among those *B. subtilis* and *B. anthracis*, were reported recently (Nicholson and Galeano 2003; Coohill and Sagripanti 2008 and references therein).

Furthermore, spores of *B. subtilis* were used as reference organisms and highly resistant, metabolically inactive cell state (reviewed in Nicholson et al. 2000, 2005; Setlow 2006) in this study to present a broader spectrum on the microbial response to UV-C radiation. The preponderance of SP in the DNA of UV-irradiated spores can be explained by different factors including the dehydrated state of the spore core, the presence of large amounts of dipicolinic acid, and the binding of small, acid-soluble spore proteins (SASP) of the α/β -type to DNA (Douki et al. 2005a, b; Moeller et al. 2007a; Setlow 2006). Binding of α/β -type SASP to the DNA of spores, together with spore core dehydration, induces a change in the helical conformation of spore DNA from the B-form to an A-like form, which in turn alters its UV-C photochemistry to favor production of SP (Nicholson et al. 2000, 2005; Setlow 2006). UV-C DNA damage induced in bacterial spores is repaired during germination, when spores reactivate and return to vegetative growth (reviewed in Setlow 2006). Studies with mutant spores have identified two DNA repair systems involved at this stage: an SP-specific repair enzyme called

Table 3 Genomic frequency of the four bipyrimidine nucleotide combinations in three tested bacterial species

Bacterial species	GC (%)	C (%)	T (%)	TT (%)	TC (%)	CT (%)	CC (%)
<i>B. subtilis</i> 168	43.51	21.80	28.30	9.89	6.58	5.64	4.60
<i>E. coli</i> K12	50.78	25.42	24.59	7.32	5.76	5.09	5.86
<i>D. radiodurans</i> R1	66.61	31.69	18.25	4.02	5.77	6.04	8.92

Fig. 3 Level of induced BPPs (TT-photoproducts, **a**; CC-photoproducts, **b**; TC-photoproducts, **c**; CT-photoproducts, **d**) as a function of the genomic bipyrimidine nucleotide frequency of *D. radiodurans* R1 (filled circles), *E. coli* K12 (filled squares), *B. subtilis* 168 cells (filled triangles down) and spores (filled triangles up; in brackets). Coefficient of determination (r^2) was 0.953 (for TT-photoproducts), 0.991 (for CC-photoproducts), 0.949 (for TC-photoproducts), and 0.179 (for CT-photoproducts) for three data sets (obtained from *D. radiodurans* R1, *E. coli* K12 and *B. subtilis* 168 cells). Data are reported as averages and standard deviations ($n = 4$). Data are averages \pm standard deviation ($n = 4$). Otherwise error bars for survival data not shown were smaller than the symbol



SP lyase that cleaves SP back to the two original thymine residues in a monomerization reaction and the nucleotide excision repair (NER) pathway that can excise SP and fill in the resulting single-strand gap (Nicholson et al. 2000, 2005). Three enzymes forming the UvrABC system initiate NER of a variety of structurally different DNA lesions, mainly BPPs caused by UV radiation exposure. UvrB (the subunit B of the excinuclease complex ABC), the central component of this system, is responsible for the ultimate DNA damage recognition and participates in the incision of the damaged DNA strand (Au et al. 2005; Goosen and Moolenaar 2008).

In bacteria, a large number of different repair mechanisms have evolved to repair UV-induced DNA lesions. These mechanisms include direct reversal of the damage by a photolyase (photoreactivation), removing of the damaged base by a DNA glycosylase (base excision repair, BER), incision of the DNA adjacent to the damage by an endonuclease (UV-damage endonuclease, UVDE) or removal of a complete oligonucleotide containing the damage by NER (reviewed in Nicholson et al. 2000; Sinha and Häder 2002; Cadet et al. 2005; Goosen and Moolenaar 2008).

The BPPs formation rates correlated highly with genomic pyrimidine dinucleotide frequencies taking into consideration the well-documented photoreactivity of each of the four primary bipyrimidine sequences (Douki and Cadet 2001, 2003; Douki et al. 2003; Cadet et al. 2005; Matallana-Surget et al. 2008; 2009). In the case of significant differences in the dinucleotide frequency, i.e. TT and CC, it can be clearly shown that an increased TT (*B. subtilis*) and CC (*D. radiodurans*) bipyrimidine nucleotide frequency led to an increased formation of TT- and CC-photoproducts, respectively, compared to the other two bacterial species with lower TT- and CC-bipyrimidine nucleotide frequency (Fig. 3a, b). However, whereas clear significant tendencies in TT- and CC-photoproduct induction were obtained, no or only very weak correlation between TC- and CT-containing photoproducts and the respective corresponding TC and CT bipyrimidine nucleotide frequency can be determined (Fig. 3c, d), which is mainly due to the fact that non-significantly different TC and CT nucleotide frequency-containing bacterial species were investigated. The results obtained in this study raise some novel questions on the relationship between genome composition and UV-mutagenesis. It would be interesting to determine how

variation in the bipyrimidine nucleotide frequencies of a genome is translated into number of bipyrimidine dinucleotides within actual genes. These data could be correlated with the distribution of DNA photolesions at the sequence level in a large series of species exhibiting different bipyrimidine nucleotide frequencies. Such information could provide some essential insight into mutagenesis caused by germicidal UV-C radiation and its consequences for the evolution of bacteria (Franklin and Haseltine 1986; Galhardo et al. 2007; Palmeira et al. 2006). Recently, Hendel et al. (2008) showed that specific BPPs such as the TT CPDs have significantly higher mutagenic properties when repaired via translesion DNA synthesis than do other BPPs, in agreement with previous data reported by Gentil et al. (1996). In chromosomal DNA from UV-C irradiated *E. coli*, Franklin and Haseltine (1986) could show CPDs are the major premutagenic lesions responsible for point mutations at sites of adjacent pyrimidine residues and were also responsible for induction of the SOS response leading to the repair and removal of this type of photolesions. Ongoing investigations on the mutagenic specificity of UV radiation-induced DNA lesions of the three microbial model systems are continuing to elucidate detailed insights into the microbial response to mutagenic events on (error-prone and error-free) DNA repair mechanisms.

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