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## Oxidative DNA Damage and Repair in the Radioresistant Archaeon *Thermococcus gammatolerans*

Ewa Barbier,<sup>†,‡</sup> Arnaud Lagorce,<sup>§,||</sup> Amine Hachemi,<sup>§</sup> Murielle Dutertre,<sup>§</sup> Aurore Gorlas,<sup>§</sup> Lucie Morand,<sup>†,‡</sup> Christine Saint-Pierre,<sup>†,‡</sup> Jean-Luc Ravanat,<sup>†,‡</sup> Thierry Douki,<sup>†,‡</sup> Jean Armengaud,<sup>⊥</sup> Didier Gasparutto,<sup>†,‡</sup> Fabrice Confalonieri,<sup>§</sup> and Jean Breton<sup>\*,†,‡</sup>

<sup>†</sup>University of Grenoble Alpes, INAC, LCIB, F-38000 Grenoble, France

<sup>‡</sup>CEA, INAC, SyMMES, F-38000 Grenoble, France

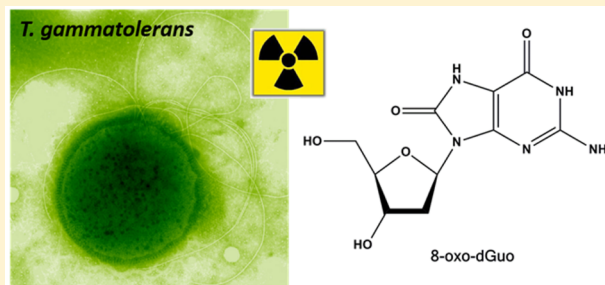
<sup>§</sup>University of Paris-Sud, Institute for Integrative Biology of the Cell (I2BC), Université Paris Saclay, CEA, CNRS, Orsay, France

<sup>||</sup>University of Perpignan, IHPE – UMR 5244 CNRS/IFREMER/Univ. Montpellier, Montpellier, F-34095, France

<sup>⊥</sup>CEA, DSV-Li2D, Laboratory “Innovative Technologies for Detection and Diagnostics”, BP 17171, Bagnols-sur-Cèze, F-30207, France

### Supporting Information

**ABSTRACT:** The hyperthermophilic archaeon *Thermococcus gammatolerans* can resist huge doses of  $\gamma$ -irradiation, up to 5.0 kGy, without loss of viability. The potential to withstand such harsh conditions is probably due to complementary passive and active mechanisms, including repair of damaged chromosomes. In this work, we documented the formation and repair of oxidative DNA lesions in *T. gammatolerans*. The basal level of the oxidized nucleoside, 8-oxo-2'-deoxyguanosine (8-oxo-dGuo), was established at  $9.2 (\pm 0.9)$  8-oxo-dGuo per  $10^6$  nucleosides, a higher level than those usually measured in eukaryotic cells or bacteria. A significant increase in oxidative damage, i.e., up to  $24.2 (\pm 8.0)$  8-oxo-dGuo/ $10^6$  nucleosides, was measured for *T. gammatolerans* exposed to a 5.0 kGy dose of  $\gamma$ -rays. Surprisingly, the yield of radiation-induced modifications was lower than those previously observed for human cells exposed to doses corresponding to a few grays. One hour after irradiation, 8-oxo-dGuo levels were significantly reduced, indicating an efficient repair. Two putative base excision repair (BER) enzymes, TGAM\_1277 and TGAM\_1653, were demonstrated both by proteomics and transcriptomics to be present in the cells without exposure to ionizing radiation. Their transcripts were moderately upregulated after gamma irradiation. After heterologous production and purification of these enzymes, biochemical assays based on electrophoresis and MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry indicated that both have a  $\beta$ -elimination cleavage activity. TGAM\_1653 repairs 8-oxo-dGuo, whereas TGAM\_1277 is also able to remove lesions affecting pyrimidines (1-[2-deoxy- $\beta$ -D-erythro-pentofuranosyl]-5-hydroxyhydantoin (5-OH-dHyd) and 1-[2-deoxy- $\beta$ -D-erythro-pentofuranosyl]-5-hydroxy-5-methylhydantoin (5-OH-5-Me-dHyd)). This work showed that in normal growth conditions or in the presence of a strong oxidative stress, *T. gammatolerans* has the potential to rapidly reduce the extent of DNA oxidation, with at least these two BER enzymes as bodyguards with distinct substrate ranges.



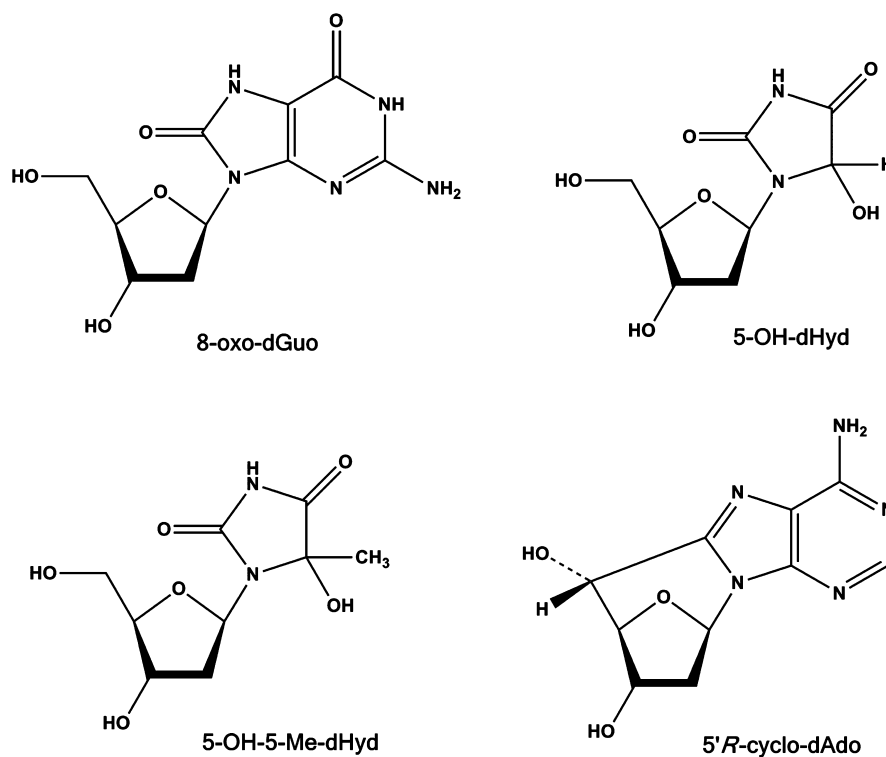
## INTRODUCTION

Extremophile microorganisms have developed a wide range of mechanisms to survive in harsh environmental conditions. *Thermococcus gammatolerans*, a hyperthermophilic prokaryote described as the most radioresistant archaeon identified to date, was isolated from samples obtained in a hydrothermal vent located in the Gulf of California.<sup>1</sup> This obligatory anaerobic heterotrophic organism has a genome of 2.04 megabases (Mb), shows an optimal growth temperature of 88 °C, and fully withstands a 5.0 kiloGrays (kGy) dose of gamma irradiation without loss of viability.<sup>2</sup> The molecular and biological mechanisms explaining this remarkable resistance of *T. gammatolerans* have not yet been defined. This microorganism probably combines different and complementary strategies to

resist such hostile conditions, as is the case for other radiotolerant organisms.<sup>3</sup> Ionizing radiation is associated with the production of reactive oxygen species (ROS), passive resistance mechanisms consist of the modification of the Mn/Fe intracellular ion ratio and the production of “compatible solutes” composed of chemical agents such as polyols, sugars, and amino acids allowing the stabilization of macromolecules.<sup>4</sup> A more active mechanism involves combating oxidative stress using antioxidant enzymes. Finally, another way to resist ionizing radiation is to repair shattered chromosomal DNA as efficiently as possible. Pulsed field gel electrophoresis has

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**Figure 1.** Chemical structures of the DNA lesions mentioned in this work. 8-oxo-dGuo, 5-OH-dHd, and 5-OH-5-Me-dHd represent single oxidation products of 2'-deoxyguanosine, 2'-deoxycytidine, and thymidine, respectively. 5'R-cyclo-dAdo represents an example of 5',8-cyclonucleosides that are considered as a family of complex oxidative DNA damage. 8-oxo-dGuo and 5',8-cyclonucleosides were measured in *T. gammatolerans* DNA at basal levels and after irradiation. 8-oxo-dGuo, 5-OH-dHd, 5-OH-5-Me-dHd, and 5',8-cyclonucleosides were tested as potential substrates for the *T. gammatolerans* enzymes, TGAM\_1653 and TGAM\_1277.

allowed time course analysis of the archaeon chromosomal repair after irradiation. Such experiments have indicated that *T. gammatolerans* chromosomes are fully degraded after a 5.0 kGy dose of  $\gamma$ -rays. DNA strand breaks are fully repaired after 5 h by cells in stationary phase, while 6 h are necessary for cells in exponential growth phase.<sup>5</sup> Proteomic approaches have demonstrated that *T. gammatolerans* produces key factors of the main DNA repair systems such as base excision repair (BER) and double-strand break repair.<sup>2</sup> It is worth noting that some of these proteins are present in the absence of stress, which may be useful for survival in the harsh conditions that constantly surround these extremophiles or during drastic environmental changes that are especially unfavorable to life. Despite a growing amount of data regarding radiotolerant organisms, DNA repair mechanisms in Archaea, and particularly in *T. gammatolerans*, remain poorly understood.

Most of the DNA alterations resulting from high doses of radiation have never been analyzed in radioresistant archaea. Among these, 8-oxo-2'-deoxyguanosine (8-oxo-dGuo) is a classic biomarker of oxidative stress. Its chemical structure is presented in Figure 1. The main mechanism of formation of 8-oxo-dGuo upon exposure to ionizing radiation depends on an indirect effect: the radiolysis of water generates the hydroxyl radical OH which reacts with C8 of the guanine.<sup>6</sup> Mutations (mainly G  $\rightarrow$  T transversions) can occur in the genome if 8-oxo-dGuo is not repaired prior to replication.<sup>7</sup> Sample preparation and measurement of 8-oxo-dGuo are technically challenging to avoid DNA artifactual oxidation, and as a consequence misleading results can be found in the literature.<sup>6</sup> DNA lesions 5',8-cyclonucleosides (Figure 1) are radiation-induced, tandem base-sugar alterations usually produced in

lower yields than 8-oxo-dGuo but with a more severe biological impact resulting from a drastic alteration of the DNA structure.<sup>8</sup> The formation of these complex DNA lesions is known to be favored at low oxygen concentrations.<sup>9</sup> The large radiation dose that can be applied to *T. gammatolerans* cells and their natural anaerobic growth conditions makes the quantitation of 5',8-cyclonucleosides highly relevant.

In the present study, we first quantified oxidative DNA damage in *T. gammatolerans*, both at basal levels and after exposure to high doses of  $\gamma$ -irradiation, by measuring 8-oxo-dGuo and 5',8-cyclonucleosides by HPLC coupled to tandem mass spectrometry (HPLC-MS/MS). We also documented the molecular mechanisms of two putative BER enzymes identified in the genome: TGAM\_1277, initially annotated as a putative type III endonuclease (endoIII), and TGAM\_1653, a predicted DNA glycosylase,<sup>2</sup> phylogenetically close to the 8-oxoguanine glycosylase from *Pyrobaculum aerophilum*.<sup>10</sup> Transcriptomics and proteomics were used to assess their presence in *T. gammatolerans* with and without exposure to irradiation. After their heterologous production, TGAM\_1277 and TGAM\_1653 proteins were assayed for their cleaving activities on different oxidative DNA lesions by electrophoresis and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry.

## EXPERIMENTAL PROCEDURES

***T. gammatolerans* Growth Conditions.** The *T. gammatolerans* EJ3 strain was cultivated in serum bottles or in 1 L Schott bottles, under anaerobic conditions at 85 °C in complex organic medium (VSM), as described in ref 2. Cell culture densities were measured by

optical microscopy (Olympus BH-29) using a Thoma counting chamber (Microgravure Precis).

**Gamma Irradiation and DNA Extraction.** *Gamma Irradiation.* For 8-oxo-dGuo quantification after irradiation, large volumes of exponentially growing cells (600 mL in 1 L Schott bottles,  $7 \times 10^7$  cells/mL) were filtered (MicraCloth Calbiochem, La Jolla, CA, USA) to remove sulfur compounds from the VSM-S<sup>o</sup> medium. In anaerobic conditions, cells were concentrated  $\times 10$  in Hungate tubes for  $\gamma$ -irradiation. Cells were then exposed on ice to a dose of 2.5 kGy or 5.0 kGy (100% survival)<sup>5</sup> using a <sup>137</sup>Cs  $\gamma$ -ray source (40 Gy/min, IBL637 CisBio International, Institut Curie, Orsay, France). As a control, the same number of nonirradiated cells was kept on ice during irradiation. Irradiated and nonirradiated cells were either collected immediately or incubated for 60 min at 85 °C for recovery in fresh VSM-S<sup>o</sup> medium on a reciprocal shaker before centrifugation (4000g, 20 min at 4 °C). Cells were immediately frozen and stored at -80 °C until further processing. Each culture was performed in independent duplicates.

For gene expression assays, 600 mL of exponentially growing cells were filtered (MicraCloth Calbiochem, La Jolla, CA, USA) to remove sulfur compounds from the VSM-S<sup>o</sup> medium and concentrated 10-fold by centrifugation. Cells were exposed on ice to a dose of 2.5 kGy or 5.0 kGy using a <sup>137</sup>Cs  $\gamma$ -ray source (19 Gy/min, GSR D1 GSM, Institut Curie, Orsay, France). As a control, the same number of nonirradiated cells was kept on ice during irradiation. Irradiated and nonirradiated cells were either collected immediately or incubated for 60 or 120 min at 85 °C for recovery in fresh VSM-S<sup>o</sup> medium on a reciprocal shaker before centrifugation (4000g, 20 min at 4 °C). Cells were immediately frozen and stored at -80 °C until further processing. Each culture was repeated twice.

**DNA Extraction and Digestion.** *T. gammatolerans* cells (10 mg) were supplemented with 300  $\mu$ L of lysis buffer containing 100 mM Tris-HCl at pH 8.3, 50 mM EDTA, 100 mM NaCl, and 0.15 mM deferoxamine. Thirty microliters of 10% SDS (v/v) and 30  $\mu$ L of 10% sarkosyl (v/v) were then added to the cell suspension. The samples were incubated with 150  $\mu$ g of RNase A (Sigma) and 3.5 U RNase T1 (Sigma) for 15 min at 50 °C. Six hundred micrograms of protease (Qiagen) was added, and the incubation was pursued for 1.5 h at 37 °C. DNA was precipitated with 600  $\mu$ L of 20 mM EDTA-Na<sub>2</sub>, 7.6 M NaI, 40 mM Tris-HCl, 0.3 mM deferoxamine at pH 8, and 1 mL of 100% isopropanol. The pellets were washed with 40% isopropanol (v/v) and subsequently with 70% ethanol (v/v), and finally suspended in 50  $\mu$ L of 0.1 mM deferoxamine.

The DNA digestion was performed in two steps. First, 0.025 U phosphodiesterase II, 2.5 U DNase II, 0.5 U nuclease P1 (Sigma), and 2.5  $\mu$ L of buffer (200 mM succinic acid and 100 mM CaCl<sub>2</sub>, pH 6) were added, and the samples were incubated for 2 h at 37 °C. Then, 6  $\mu$ L of 500 mM Tris-HCl, 1 mM EDTA at pH 8, 0.015 U phosphodiesterase I (Worthington), and 2 U alkaline phosphatase (Sigma) were added, and the samples were incubated for 2 h at 37 °C. The reaction was stopped by adding 3.5  $\mu$ L of 0.1 N HCl. For cyclonucleoside detection, the first step of the digestion was repeated before adding HCl, as optimized previously.<sup>9</sup>

**HPLC-MS/MS Quantification of 8-Oxo-dGuo and 5',8-Cyclonucleosides.** *8-Oxo-dGuo.* Digested DNA samples were resolved on an Uptisphere 2  $\times$  150 mm octadecylsilyl silica gel column (Interchim) operated with an Agilent series 1100 HPLC system. The mobile phase was a gradient of methanol in 2 mM aqueous ammonium formate (2 to 10% (v/v) in 25 min and 10 to 25% (v/v) in the following 10 min). Undamaged nucleosides were monitored with a UV detector set at 260 nm, while 8-oxo-dGuo was measured in positive mode with an API 3000 triple quadrupole mass spectrometer (PerkinElmer/SCIEX) equipped with electrospray ionization (ESI). The system was operated in multiple reaction monitoring mode using a specific transition ( $m/z$  284  $\rightarrow$  168). DNA damage rates measured in different experimental conditions were compared using the Mann-Whitney test ( $p < 0.05$ ). For each condition, five measurements were performed: two technical replicates for the first biological sample and three technical replicates for the second biological sample.

**5',8-Cyclonucleosides.** Four lesions were measured simultaneously: 5'R and 5'S diastereomers of 5',8-cyclo-2'-deoxyadenosine (5',8-

cyclodAdo) and 5',8-cyclo-2'-deoxyguanosine (5',8-cyclodGuo). Digested DNA samples were resolved on an Uptisphere 2  $\times$  150 mm column of silica octadecylsilyl (Interchim) operated with an Accela HPLC (Thermo Corporation). Nucleosides were resolved by a linear gradient of acetonitrile (0 to 15% (v/v) gradient in 30 min) in ammonium formate. Undamaged nucleosides were detected at 260 nm with a UV detector, while cyclonucleosides were specifically monitored as previously described<sup>9</sup> with a TSQ Quantum Ultra mass detector equipped with ESI.

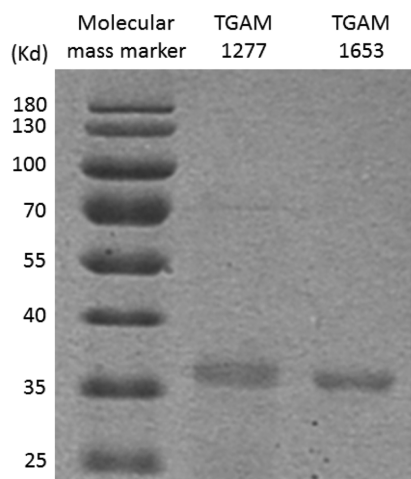
**Transcriptomic and Proteomic Analysis of TGAM\_1277 and TGAM\_1653.** For expression profiling by real-time RT-PCR (reverse transcription PCR), total RNAs were extracted with TriReagent solution (Sigma), and genomic DNA was digested by Turbo DNase (Ambion) according to the manufacturer's instructions. The absence of genomic DNA in RNA samples was checked by PCR using 16S rRNA primers (Table S1) and 2  $\mu$ g of total RNA as template. Reverse transcription was performed in a total volume of 20  $\mu$ L in the presence of 5  $\mu$ g of total RNA, 2 pmol gene-specific reverse primers (Table S1), 0.5 mM each dNTP, and 200 units of SuperScriptII (Invitrogen, Carlsbad, CA). After a 30 min of incubation at 42 °C, 200 units of SuperScriptII was added, and the samples were incubated at 42 °C for 30 min. The hydrolysis of the RNA was performed in the presence of 11  $\mu$ L of 1 N NaOH for 10 min at 70 °C, and the solution was neutralized with 11  $\mu$ L of 1 N HCl. cDNAs were desalted with Microcon-G30 (Millipore), dried under vacuum, and resuspended in 10  $\mu$ L of water. cDNA templates were diluted 1/100 with water for expression measurements. Standard conditions were used with LightCycler FastStart DNA MasterPLUS SYBR green I (Roche), i.e., DNA denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s, using the LightCycler System (Roche Diagnostics). Gene expression was calculated relative to the geometric average expression of four reference genes using the formula  $2^{-\Delta Ct}$ .<sup>11,12</sup> The reference genes used in this work, namely, TGAM\_0829, TGAM\_01054, TGAM\_1165, and TGAM\_1926, encode a TATA box-binding protein (tbp), an elongation factor 1-alpha (tuf), a CDP-alcohol phosphatidyltransferase, and a DNA-directed RNA polymerase subunit B (rpoB),<sup>13</sup> respectively. Expression of these selected reference genes remains particularly stable in *T. gammatolerans* cells challenged with heavy metal (i.e., cadmium)<sup>13</sup> or with ionizing radiation, as revealed by genome-wide transcriptomic data (unpublished data). Proteomics information for TGAM\_1277 and TGAM\_1653 were extracted from data we had previously obtained through a shotgun proteomic analysis, now available on the ProteomeXchange Consortium (data set identifier PXD000402).

**Heterologous Production and Purification of BER Enzymes.** *Bacterial Strains and Expression Plasmids.* *E. coli* strain DH5 $\alpha$  (Life Technologies) was used in all cloning experiments and for plasmid amplifications. The *E. coli* Rosetta (DE3) strain (Novagen) was used for protein production. The pET30a(+) plasmid (Novagen) was used for bacterial expression of N-terminal His<sub>6</sub>-tagged proteins.

**Cloning and Expression of TGAM\_1277 and TGAM\_1653 Glycosylase Genes.** TGAM\_1277 (230 aa) and TGAM\_1653 (263 aa) coding DNA sequences (CDS) were first amplified by PCR with genomic DNA as the template using a high fidelity DNA polymerase (Fusion high fidelity, ThermoFisher Scientific), according to the manufacturer's instructions (DNA denaturation at 95 °C for 2 min followed by 25 cycles of 95 °C for 10 s, 55 °C for 20 s and 72 °C for 30 s) and using primer pairs containing *EcoRV* and *XhoI* restriction sites at their 5' and 3' ends, respectively, as described in Table S2. The PCR fragments were digested with the corresponding restriction enzymes and cloned in pET30a(+) at the same unique restriction sites found in the polylinker. The amplified CDS and their fusion in-frame with the series of tags located in the N-terminal of the recombinant proteins, including a His<sub>6</sub>-tag, were confirmed by double-stranded sequencing using T7P and T7TERM primers (Beckman Coulter Genomics). Because of the codon usage bias in archaea, both proteins were expressed in the *E. coli* Rosetta (DE3) strain encoding rare tRNA genes, a strategy which has proved successful for numerous archaeal proteins.<sup>14,15</sup> *E. coli* Rosetta (DE3) strain cells were transformed with the individual expression plasmid and were grown overnight at 37 °C

with shaking in 5 mL of LB media containing 25  $\mu\text{g}/\text{mL}$  kanamycin and 30  $\mu\text{g}/\text{mL}$  chloramphenicol. The cultures were transferred into 500 mL (1:100 dilution) of LB media supplemented with 25  $\mu\text{g}/\text{mL}$  kanamycin and 30  $\mu\text{g}/\text{mL}$  chloramphenicol in 2 L Erlenmeyer flasks until the  $\text{OD}_{600}$  reached 0.5–0.6. The His<sub>6</sub>-tagged TGAM\_1277 and TGAM\_1653 recombinant proteins were expressed for 4 h at 37 °C after induction with 1 mM IPTG (isopropyl  $\beta$ -D-1-thiogalactopyranoside). Cells were harvested by centrifugation and resuspended in 8 mL of buffer A (20 mM Tris-HCl, pH 7.5, 1 mM NaCl, 1 mM DTT, and 20 mM imidazole), supplemented with 300  $\mu\text{L}$  of a protease inhibitor cocktail for bacterial cells (Sigma-Aldrich), and finally stored at –80 °C overnight.

**Purification of TGAM\_1277 and TGAM\_1653 Recombinant Glycosylases.** After thawing on ice, *E. coli* cells were lysed with an Eaton Press at a pressure of 10,000 psi, and the resulting extracts were sonicated for 2  $\times$  15 s. The protein extracts were centrifuged at 15,000g for 30 min at 4 °C. The soluble proteins were loaded onto His-trap columns (Macherey Nagel) previously equilibrated with buffer A and washed in the same buffer. The recombinant proteins were purified by affinity FPLC (fast protein liquid chromatography) (Amersham, GE Healthcare) with increasing steps of imidazole (50 mM, 150 mM, and 250 mM) in the same buffer and dialyzed twice (Spectrumlabs, MWCO 15 kDa) overnight at 4 °C against 500 mL of buffer B (20 mM Tris-HCl, pH 7.5, 50% Glycerol v/v, 200 mM NaCl, and 1 mM DTT). The purified recombinant proteins were almost 95% pure as judged by SDS–PAGE analysis (Figure 2). The concentrations of TGAM\_1653 and TGAM\_1277 were 0.33 and 0.22 mg/mL, respectively, as estimated by Bradford assays.



**Figure 2.** Purification of recombinant TGAM\_1277 and TGAM\_1653 by FPLC affinity chromatography on His-Trap columns after expression in *E. coli*. TGAM\_1277 (4.8  $\mu\text{g}$ ) and TGAM\_1653 (5.1  $\mu\text{g}$ ) were resolved on a 10% SDS–PAGE gel and stained with Instant Blue (Expedeon).

**DNA Glycosylase Activity Assays. Oligonucleotides (ODNs).** In order to perform activity tests, ODNs of the following sequences were used: 5'-CAC TTC GG<sup>1</sup>A T<sup>2</sup>CG TGA CTG ATC T-3' (control), where G<sup>1</sup> was replaced by 8-oxo-dGuo, and T<sup>2</sup> by 1-[2-deoxy- $\beta$ -D-erythro-pentofuranosyl]-5-hydroxyhydantoin (5-OH-dHyd) or 1-[2-deoxy- $\beta$ -D-erythro-pentofuranosyl]-5-hydroxy-5-methylhydantoin (5-OH-5-Me-dHyd). All ODNs, except those containing 8-oxo-dGuo (Eurogentec), were synthesized in-house as previously described.<sup>16,17</sup> In order to form double stranded ODNs, sequences fully complementary to the control ODN were obtained from Eurogentec, along with three other ODNs presenting G, A, or T mismatches opposite the G<sup>1</sup> position.

**Radiolabeling of ODNs.** ODNs (10 pmol) were [<sup>5'</sup>-<sup>32</sup>P]-labeled at their 5'-ends with 10  $\mu\text{Ci}$  [<sup>32</sup>P] ATP (10 mCi/mL, PerkinElmer France) and 1 unit of T4 polynucleotide kinase (10 U/ $\mu\text{L}$ , Life Technologies/Invitrogen) in the supplied "forward" reaction buffer.

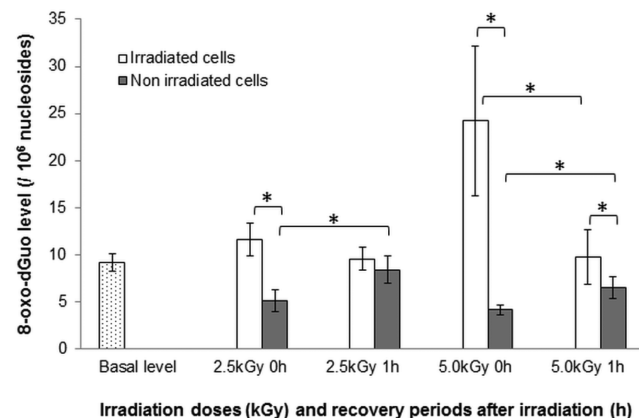
The labeling reaction was performed at 37 °C for 1 h. Unincorporated [<sup>32</sup>P] ATP was then removed using Illustra MicroSpin G-25 Columns (GE Healthcare), and the [<sup>5'</sup>-<sup>32</sup>P]-labeled strands were finally hybridized with their complementary sequences by incubation at 90 °C for 3 min followed by slow cooling until room temperature was reached.

**Polyacrylamide Gel Electrophoresis.** The [<sup>5'</sup>-<sup>32</sup>P]-labeled ODN duplexes (100 fmol) were incubated with 2 pmol TGAM\_1653 or TGAM\_1277 proteins in the reaction buffer consisting of 20 mM Tris-HCl, buffered at pH 7.8, and containing 1 mM EDTA and 150 mM KCl. After 30 min of incubation at 37 °C, the temperature was raised progressively to 65 °C, and the reaction was maintained at this temperature for 10 min. In parallel, control experiments with *E. coli* formamidopyridine DNA N-glycosylase (Fpg) (a gift from Dr. B. Castaing, CNRS, Orléans, France) were performed. An incubation of 1 h at 37 °C was carried out in the reaction buffer for Fpg. The samples were then denatured by heating for 3 min at 90 °C in formamide solution and resolved by electrophoresis on polyacrylamide gel in denaturing conditions. Signal detection was performed by phosphor-imaging with the Personal Molecular Imager system (Bio-Rad).

**MALDI-TOF-MS.** For MALDI-TOF-MS measurements, ODNs were not radioactively labeled but directly hybridized with their complementary sequence. These duplexes (10 pmol) were incubated with 20 pmol TGAM\_1653, TGAM\_1277, Fpg, or Endo III proteins, in conditions as described above for TGAM\_1653 and TGAM\_1277. The samples were then desalted with the 50WX8 100–200 Dowex resin (Sigma-Aldrich) and analyzed with a Microflex instrument (Bruker).

## RESULTS

**Basal Levels of Oxidative DNA Damage and Quantitation after Irradiation.** We first wanted to obtain quantitative data about oxidative DNA damage in *T. gammatolerans* with and without exposure to ionizing radiation. DNA damage was measured in cells that were neither irradiated nor incubated on ice ("basal levels", Figure 3). Cells were also exposed to a  $\gamma$ -ray dose of either 2.5 or 5.0 kGy. The exposure lasted 62.5 min (2.5 kGy) or 125 min (5.0 kGy) and was performed on ice to avoid a significant increase of temperature. Cells were then either immediately collected after irradiation or



**Figure 3.** Levels of 8-oxo-dGuo in *T. gammatolerans*. The "basal level" of 8-oxo-dGuo was determined with cells that were neither irradiated nor kept on ice. For gamma irradiation experiments performed at 2.5 kGy or 5.0 kGy, cells were harvested immediately after treatment (0 h) or after 1 h of recovery in culture media (1 h). Nonirradiated controls were stored on ice, while the other cells were irradiated. Vertical bars:  $\pm$  SD. For each condition, five measurements were performed: two technical replicates for the first biological replicate and three technical replicates for the second biological replicate. \*: statistically significant difference, according to the Mann–Whitney test, with  $p < 0.05$ .

incubated in fresh medium for 1 h before being processed. For each condition, nonirradiated controls were prepared in parallel and stored on ice during irradiation. Extraction protocols were carefully optimized, on the basis of a protocol developed for mammalian cells,<sup>18</sup> to obtain DNA solutions suitable for HPLC-MS/MS measurement of nucleosides and to avoid artifactual oxidation of DNA, which may lead to an overestimation of damage levels.

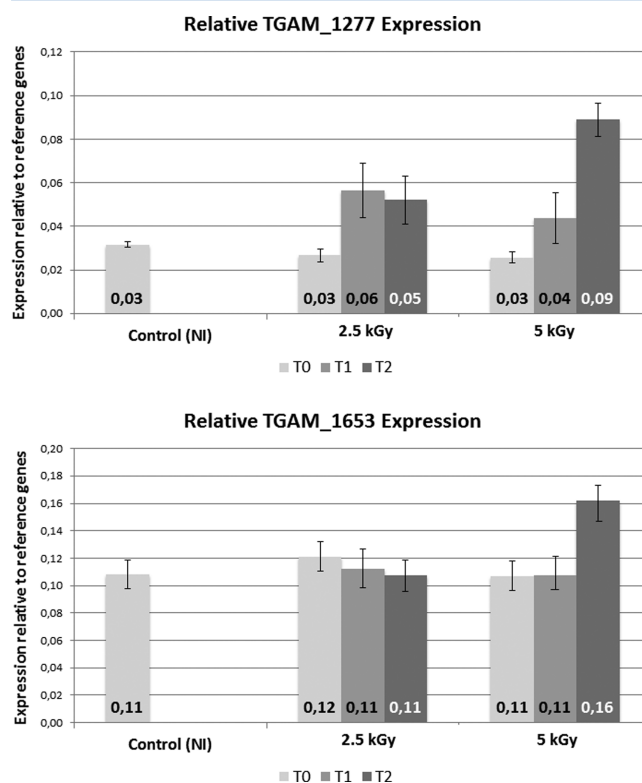
In these conditions, 8-oxo-dGuo basal levels in *T. gammatolerans* ( $9.2 \pm 0.9$  8-oxo-dGuo/ $10^6$  nucleosides) were more abundant than that in nonirradiated cells stored on ice, which presented a minimum of  $4.2 \pm 0.5$  8-oxo-dGuo/ $10^6$  nucleosides for the 5.0 kGy/0 h control and a maximum of  $8.4 \pm 1.4$  8-oxo-dGuo/ $10^6$  nucleosides for the 2.5 kGy/1 h control. Irradiated cells exhibited higher levels of DNA lesions than the corresponding controls (Figure 3), particularly at 5.0 kGy, with an increase from  $4.2 \pm 0.5$  8-oxo-dGuo/ $10^6$  nucleosides for controls to  $24.2 \pm 8.0$  8-oxo-dGuo/ $10^6$  nucleosides for irradiated cells ( $p < 0.05$ , Mann–Whitney). The 1 h incubation of irradiated cells in a fresh medium was associated with a decrease in 8-oxo-dGuo mean values, which was only significant after 5.0 kGy treatment, falling to  $9.7 \pm 2.9$  8-oxo-dGuo/ $10^6$  nucleosides. In control cells, incubation in fresh medium was associated with a slight but statistically significant increase of oxidized purines: from  $5.1 \pm 1.2$  8-oxo-dGuo/ $10^6$  nucleosides to  $8.4 \pm 1.4$  8-oxo-dGuo/ $10^6$  nucleosides for 2.5 kGy controls and from  $4.2 \pm 0.5$  8-oxo-dGuo/ $10^6$  nucleosides to  $6.5 \pm 1.1$  8-oxo-dGuo/ $10^6$  nucleosides for 5.0 kGy controls.

The 5',8-cyclonucleosides (5'R and 5'S diastereomers of 5',8-cyclodAdo and 5',8-cyclodGuo) were also measured in the same experimental conditions, but chromatograms resulting from these analyses did not show detectable peaks, indicating the absence of these DNA alterations or rates below the limit of detection of our methods, which, during this experiment, was estimated at 1.0 lesion/ $10^6$  nucleosides.

**Proteomic Detection of TGAM\_1277 and TGAM\_1653, and Transcriptional Expression of the Corresponding Genes.** We focused on two putative BER enzymes identified in the genome of *T. gammatolerans*: TGAM\_1277 and TGAM\_1653, which have been annotated as a putative endoIII and a predicted 8-oxoG glycosylase, respectively. We analyzed their occurrence and possible regulation in cells by combining proteomic and transcriptomic approaches. A recent shotgun proteomic survey has been conducted for *T. gammatolerans* cells subjected to a 5.0 kGy  $\gamma$ -irradiation dose and the corresponding control without irradiation.<sup>19</sup> We extracted from the data deposited to the ProteomeXchange Consortium (data set identifier PXD000402) the spectral counts for the TGAM\_1277 and TGAM\_1653 proteins in order to evaluate the presence of both proteins. The former was detected and validated through two proteotypic peptides ([134-CANIVLAYGFGR-145] and [215-ELCPYAKGLVR-225]), while the latter was detected by means of a unique peptide ([217-AEETGIPPLHIDSILWPVLGGK-239]) in this shotgun survey. As estimated by the normalized spectral abundance factor (NSAF) obtained by normalizing spectral counts with the molecular weight of each protein,<sup>20</sup> TGAM\_1277 (NSAF 0.22) and TGAM\_1653 (NSAF 0.10) were slightly less abundant compared to TGAM\_1679, the reverse gyrase (NSAF 0.95), and in any case present in fairly low amounts compared to the whole set of detected proteins.

We monitored by real-time RT-PCR the expression of their corresponding genes, TGAM\_1277 and TGAM\_1653, in

control conditions and following  $\gamma$ -irradiation. For  $\gamma$ -irradiation, exponentially growing cells were exposed to 2.5 or 5.0 kGy and collected immediately (T0) or incubated postirradiation in fresh VSM medium at 85 °C for 60 or 120 min (T1 and T2, see Experimental Procedures). At both doses, 100% survival had previously been observed.<sup>5</sup> Expression profiles of both genes in these conditions are shown in Figure 4. For TGAM\_1277, we observed a comparable relative



**Figure 4.** Expression profiles of TGAM\_1277 and TGAM\_1653 in *T. gammatolerans* cells exposed to  $\gamma$ -rays at a dose of 2.5 and 5.0 kGy. TGAM\_1277 and TGAM\_1653 expression were quantified by real-time RT-PCR using RNA extracted from control cells (not irradiated: NI), cells collected immediately after irradiation (T0), and cells incubated for 1 or 2 h post-irradiation (T1; T2). TGAM\_1277 and TGAM\_1653 expression were calculated relative to the transcript levels of constitutively expressed genes (TGAM\_0829, TGAM\_01054, TGAM\_1165, and TGAM\_1926) according to the formula:  $2^{-\Delta\Delta C_t} = 2^{-(C_{\text{GeneX}} - C_{\text{RefGenes}})}$ . Each data point is the average of two biological replicates. The standard deviation is indicated by error bars. The TGAM\_1277 and TGAM\_1653 genes were expressed in all conditions tested.

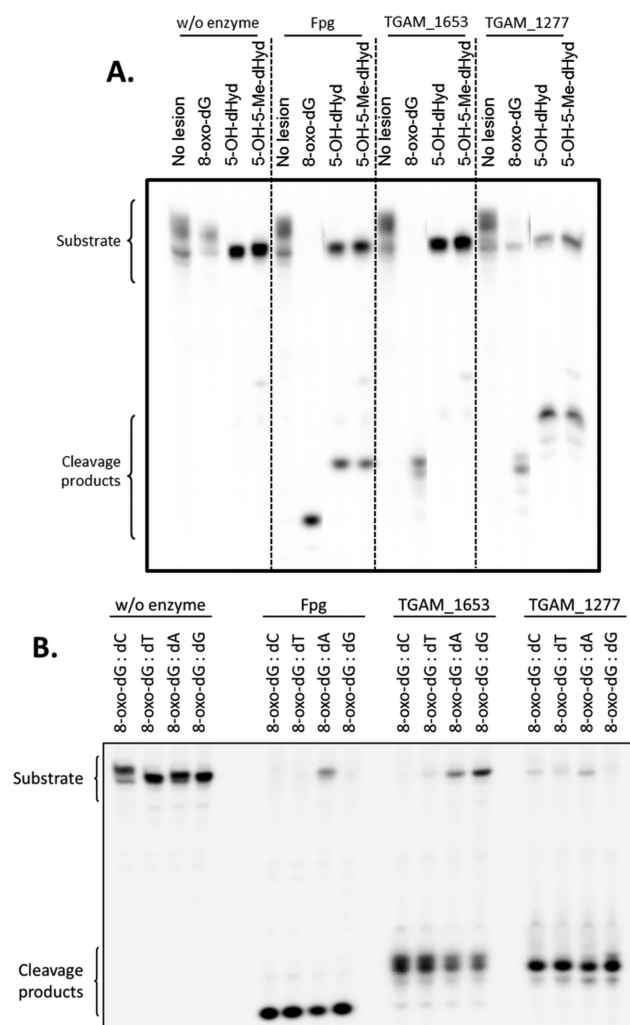
expression in control conditions and in cells immediately collected after irradiation (T0; 0.03). A slight increase (2-fold) of the relative expression could be detected 1 and 2 h (T1, T2) after a dose of 2.5 kGy. Interestingly, exposure to a higher dose (5.0 kGy) led to a significant upregulation (3-fold) as measured 2 h after irradiation (T2). For TGAM\_1653, a similar relative expression was revealed when nonirradiated cells and cells collected immediately after irradiation were compared (0.11–0.12). Our data show that the basal relative expression of TGAM\_1653 (0.11–0.12) was higher than that with TGAM\_1277 (0.03). However, TGAM\_1653 relative expression remained stable following  $\gamma$ -irradiation at a 2.5 kGy dose, and only a weak upregulation could be measured after 2 h (T2) for 5.0 kGy irradiation.

**Activity of Recombinant TGAM\_1653 and TGAM\_1277.** To characterize their biochemical activities, the two glycosylases were produced in *Escherichia coli* in fusion with a His<sub>6</sub>-tag in the N-terminal domain of the protein, as several glycosylases contain a zinc finger motif in their C-terminal domain. We decided to not remove the 48-amino-acid N-terminal extension by proteolysis as our data showed that it does not disrupt protein activities (see hereafter). The excision activities of the recombinant TGAM\_1653 and TGAM\_1277 proteins were assessed at a temperature increasing from 37 to 65 °C by means of an electrophoretic cleavage assay using double-stranded and <sup>32</sup>P-labeled ODNs. DNA lesions inserted into the substrates were (i) 8-oxo-dGuo, which is a model of oxidized purine, (ii) 5-OH-dHyd, and (iii) 5-OH-5-Me-dHyd, the latter two being models of oxidized pyrimidines. For this experimental setup, the bacterial Fpg enzyme, which is known to have a glycosylase activity toward 8-oxo-dGuo, 5-OH-dHyd, and 5-OH-5-Me-dHyd, was used as a positive control.

TGAM\_1653 was only able to cleave probes containing 8-oxo-dGuo, whereas TGAM\_1277 showed a broader substrate specificity by reacting with the three lesions (Figure 5A). The effect of the opposite base on glycosylase activity toward 8-oxoG (8-oxo-7,8-dihydroguanine, the oxidized base) was also evaluated using four probes with the four different bases in front of the altered position (Figure 5B). As already shown by Tchou et al.,<sup>21</sup> Fpg activity is partially inhibited by an 8-oxoG:A mismatch. Once again, TGAM\_1277 presented a broad activity in contrast to TGAM\_1653, the latter being less efficient when confronted with DNA containing 8-oxo-G:A and 8-oxo-G:G pairs, whereas TGAM\_1277 activity was only weakly affected by the opposite base of the lesion.

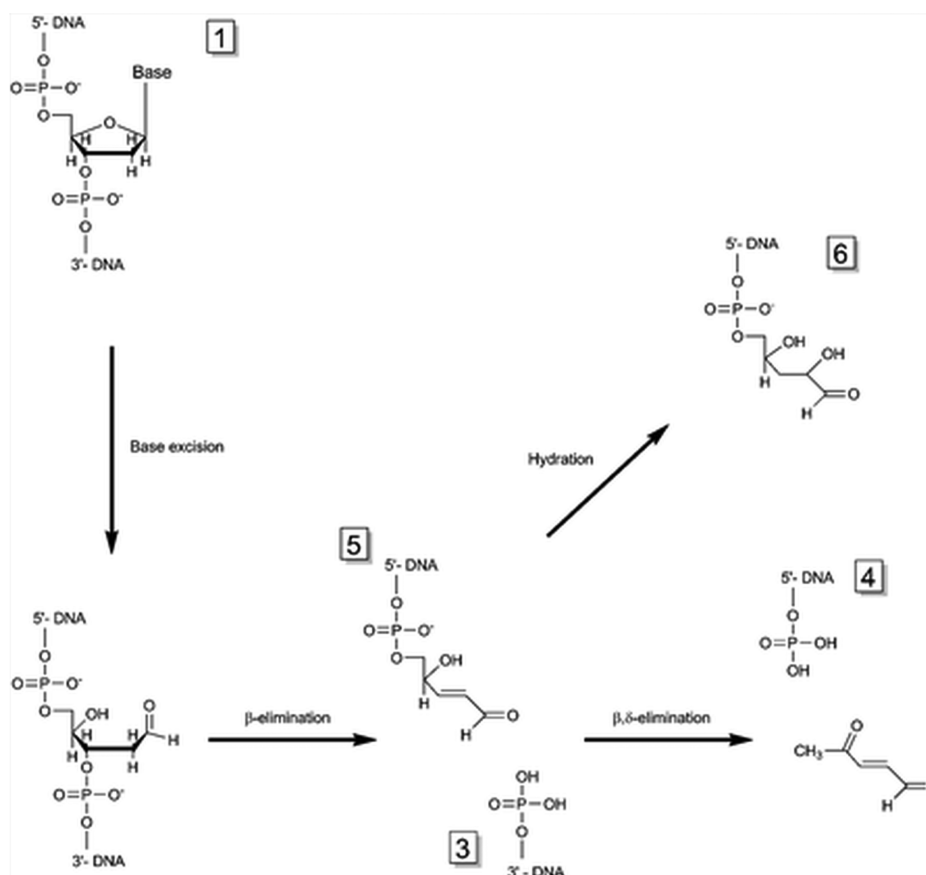
The gels in Figure 5A display different sizes of short excision products. For a given enzyme tested for different lesions, this phenomenon is simply explained by the different localizations of DNA modifications within the probe sequences. However, this difference in migration was also noted for one given lesion cleaved by different enzymes: fragments generated by TGAM\_1277 with hydantoin lesions exhibited higher molecular weights than others produced with Fpg. Regarding 8-oxo-dGuo, fragments obtained with TGAM\_1653 and TGAM\_1277 were also of higher molecular weight than the cleavage products of Fpg. As this observation could probably be explained by differences in excision mechanisms, we used MALDI-TOF MS to obtain further insight. This methodology allows a precise determination of the size of the cleavage products, hence providing complementary details about repair mechanisms.<sup>22</sup> Regarding the excision process of both *T. gammatolerans* enzymes, our hypothesis was that a  $\beta$ -elimination occurs, while in contrast Fpg is known to act as a  $\beta,\delta$ -elimination enzyme.<sup>23</sup> Both possible elimination processes are described in Figure 6.

In our experiments, Fpg and endoIII were used as controls for  $\beta,\delta$ -elimination of 8-oxo-dGuo and  $\beta$ -elimination of 5-OH-5-Me-dHyd, respectively. Experimental MALDI-TOF MS spectra acquired on the products of Fpg and recombinant TGAM\_1653 and TGAM\_1277 excision are shown in Figures 7 and 8. 8-oxo-dGuo repair by Fpg was first characterized by the absence of peak 1 corresponding to 8-oxo-dGuo-containing strands. This was indicative of a total cleavage of the substrate by the enzyme. The presence of peaks 3 ( $[M - H]^- = 4332.9$  Da; calculated = 4332.8 Da) and 4 ( $[M - H]^- = 2135.6$  Da; calculated = 2135.4 Da) is characteristic of the  $\beta,\delta$ -elimination process. For TGAM\_1653 tested on 8-oxo-dGuo substrates,

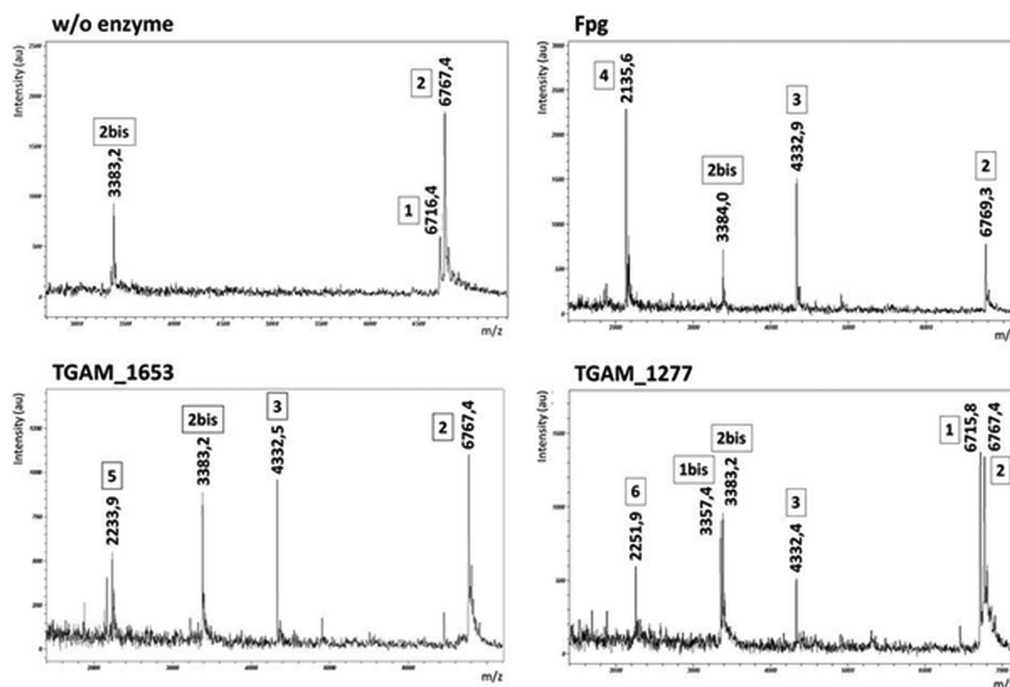


**Figure 5.** (A) Base excision activities of Fpg, TGAM\_1653, and TGAM\_1277 assessed with three oxidative DNA lesions: 8-oxo-dGuo, 5-OH-dHyd, and 5-OH-Me-dHyd. 22-mer ODN sequence: 5'-CAC TTC GG<sup>A</sup> T<sup>2</sup>CG TGA CTG ATC T-3' (control). In modified ODNs, G<sup>1</sup> was replaced by 8-oxo-dGuo and T<sup>2</sup> by 5-OH-dHyd or 5-OH-5-Me-dHyd. 100 fmol ODNs were incubated with 2 pmol enzyme. For Fpg, the cleavage was carried out at 37 °C. For TGAM\_1653 and TGAM\_1277, the incubation was initiated at 37 °C, and the temperature was progressively increased to 65 °C. The different sizes of the cleavage products are explained by the positions of the damaged bases in the ODN sequences and by the enzymes presenting different excision processes. (B) Base excision activities of Fpg, TGAM\_1653, and TGAM\_1277 on probes containing 8-oxo-dGuo. The influence of the opposite nucleoside was evaluated by using, for each enzyme, a set of four ODNs with dC, dT, dA, or dG paired with the oxidative lesion.

the absence of peak 1 proves a complete cleavage, whereas the presence of peaks 3 ( $[M - H]^- = 4332.5$  Da) and 5 ( $[M - H]^- = 2233.9$  Da; calculated = 2233.4 Da) confirms a  $\beta$ -elimination mechanism. The MALDI-TOF MS spectrum of TGAM\_1277 activity revealed that peak 1 was clearly visible, indicating that in our experimental conditions, this enzyme did not cut all the available substrate. TGAM\_1277 was therefore considered as less active than Fpg and TGAM\_1653, confirming the results obtained with electrophoretic gels (Figure 5). Considering the cleavage products, we were able to detect peak 3 but not peak 4, thus characterizing a  $\beta$ -elimination process. Actually, the presence of peak 6 ( $[M -$

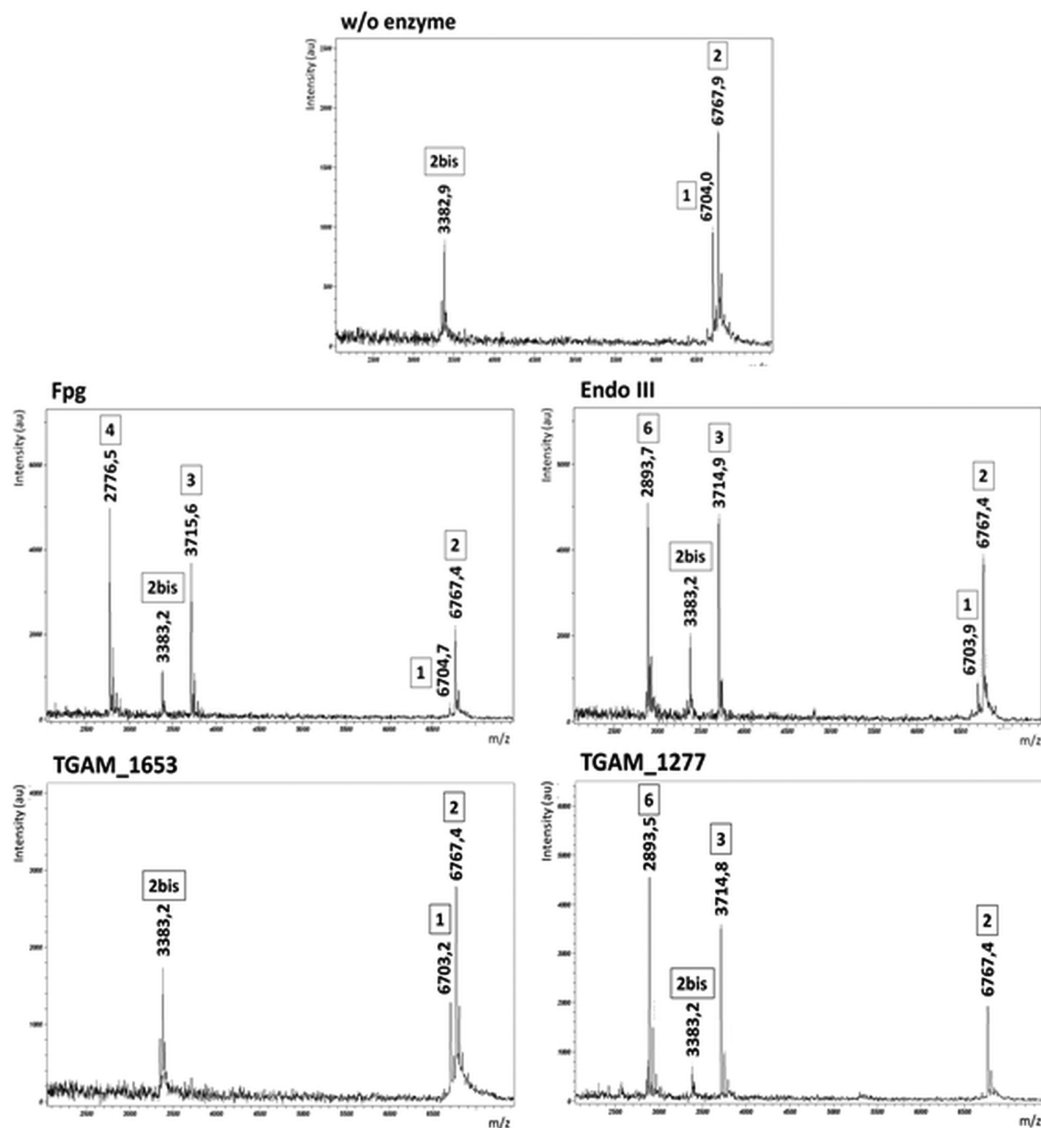


**Figure 6.** Excision reaction catalyzed by bifunctional glycosylases via  $\beta$  or  $\beta, \delta$ -elimination mechanisms. Successive cleavages of 3' and 5'-strands on each side of the abasic site lead to deoxyribose residue excision. Numbers 1 to 6 were attributed to each reaction product to match with fragments obtained by MALDI-TOF mass spectrometry analysis (Figures 7 and 8). Number 2 (not represented) stands for the complementary strand.



**Figure 7.** MALDI-TOF MS spectra resulting from the excision by Fpg, TGAM\_1653, or TGAM\_1277 of ODNs containing 8-oxo-dGuo. Ten pmol ODNs were incubated with 20 pmol enzyme. The incubation was initiated at 37 °C, and the temperature was progressively increased to 65 °C. The nature of the detected fragments (from 1 to 6) is described in Figure 6. "bis" indicates a secondary ion presenting two charges.





**Figure 8.** MALDI-TOF MS spectra resulting from the excision by Fpg, Endo III, TGAM\_1653, or TGAM\_1277 of ODNs containing 5-OH-5-Me-dHyd. Ten pmol ODNs were incubated with 20 pmol enzyme. The incubation was initiated at 37 °C, and the temperature was progressively increased to 65 °C. The nature of the detected fragments (from 1 to 6) is described in Figure 6. “bis” indicates a secondary ion presenting two charges.

$\text{H}]^- = 2251.9 \text{ Da}$ ; calculated = 2251.5 Da) probably reveals a hydration product of the  $\alpha$ - $\beta$ -unsaturated ketone, which should have been represented by fragment 5.<sup>24</sup> We can therefore assume that TGAM\_1277 uses a  $\beta$ -elimination process.

The same principle was applied to the cleavage of ODNs containing 5-OH-5-Me-dHyd (Figure 8). The MALDI-TOF MS profile corresponding to our control endoIII confirms its  $\beta$ -elimination activity with the presence of peak 3 ( $[\text{M} - \text{H}]^- = 3714.9 \text{ Da}$ ; calculated = 3715.4 Da) and hydrated product 6 ( $[\text{M} - \text{H}]^- = 2893.7 \text{ Da}$ ; calculated = 2893.9 Da). The  $\beta$ , $\delta$ -elimination exhibited by Fpg is demonstrated again by the presence of peaks 3 ( $[\text{M} - \text{H}]^- = 3715.6 \text{ Da}$ ; calculated = 3715.4 Da) and 4 ( $[\text{M} - \text{H}]^- = 2776.5 \text{ Da}$ ; calculated = 2777.8 Da). The MS profile of TGAM\_1277 is very close to that obtained with endoIII: product 3 at 3714.8 Da and product 6 at 2893.5 Da indicating  $\beta$ -elimination. The only difference with endoIII is the absence of peak 1, indicating a more efficient cleavage. TGAM\_1653 is not associated with short fragments, a

characteristic that again indicates its absence of activity on 5-OH-5-Me-dHyd.

In conclusion, TGAM\_1653 and TGAM\_1277 both exhibit a  $\beta$ -elimination cleavage activity. TGAM\_1653 is only able to cleave 8-oxo-dGuo, whereas TGAM\_1277 is also able to cut ODNs containing pyrimidine lesions (5-OH-dHyd and 5-OH-5-Me-dHyd). It is worth noting that ODN substrates containing 8-oxodA and 5',8-cyclonucleosides were also tested but did not show detectable excisions (data not shown).

## DISCUSSION

***T. gammatolerans* Shows a Basal Level of 8-Oxo-dGuo That Is Enhanced by High Doses of  $\gamma$ -Irradiation but Rapidly Decreases Postexposure.** Measurement of oxidative base damage in cellular DNA is a rather commonly used biomarker of oxidative stress but has for a long time been associated with questionable results due to possible overestimation related to technical artifacts. By using the most appropriate preanalytical precautions, as well as sensitive and

specific methods such as HPLC-electrochemical detection and, more recently, HPLC-MS/MS, it is now accepted that background levels of 8-oxo-dGuo in human cells such as lymphocytes are below the threshold of 1 lesion per  $10^6$  nucleosides (4 lesions per  $10^6$  guanines).<sup>25,26</sup> This level corresponds to approximately 10,000 8-oxo-dGuo per single nucleus.<sup>27</sup> Using HPLC-MS/MS, 8-oxo-dGuo measurements in *E. coli* DNA carried out by our team showed basal rates of 2.6 ( $\pm 0.9$ ) 8-oxo-dGuo per  $10^6$  nucleosides (unpublished data). In the present study, basal levels measured in *T. gammatolerans* grown in laboratory experimental conditions were 9.2 ( $\pm 0.9$ ) 8-oxo-dGuo per  $10^6$  nucleosides, which represents approximately 18 8-oxo-dGuo per chromosome (*T. gammatolerans* is a polyploid organism for which the mean number of chromosome copies has not been determined yet). While an overwhelming proportion of published studies reporting 8-oxo-dGuo rates have been performed using mammalian cells, the only relevant work comparable with ours was carried out using the archaeon *Halobacterium salinarum*.<sup>28</sup> In this halophilic organism, a basal level of 2.1 8-oxoG (oxidized base) per  $10^6$  bases was observed by means of gas chromatography coupled to mass spectrometry. Two other oxidative purine lesions were assessed in the same study: 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), which showed concentrations of 0.3 and 0.9 lesion per  $10^6$  bases, respectively. Elevated rates of 8-oxo-dGuo found in *T. gammatolerans* could be related to the growth conditions and more specifically to the temperature. Indeed, the optimal growth temperature for this hyperthermophilic organism (85 °C) was chosen for the experimental work presented here, while lower temperatures were used in other studies: 37 °C for human cells and 42 °C for *H. salinarum*. Heat-induced formation of oxidized guanine, at least *in vitro*, has been reported.<sup>29</sup> Because nonirradiated cells stored on ice presented only 4.2 8-oxo-dGuo lesions per  $10^6$  nucleosides, this hypothesis of heat-induced damage formation is reinforced. This was confirmed by the result obtained with nonirradiated cells incubated for 1 h at 85 °C in culture medium, which also showed a significant increase in their 8-oxo-dGuo content. It can be highlighted here that another type of DNA damage, abasic sites, was also found at elevated levels in *Pyrococcus abyssi*: between 25 (exponential phase) and 42 (stationary phase) abasic sites per 100,000 base pairs in the genome of this hyperthermophilic archaeon versus 2 and 4, respectively, abasic sites per 100,000 base pairs in *E. coli*.<sup>30</sup>

In order to study DNA damage in the context of exposure to ionizing radiation, *T. gammatolerans* samples were subjected to high doses of  $\gamma$ -radiation. Levels of 8-oxo-dGuo measured in cells harvested immediately after treatment significantly increased. The corresponding yields of radiation-induced modifications are 2.8 8-oxo-dGuo/ $10^{12}$  nucleosides/Gy at 2.5 kGy and 4.0 8-oxo-dGuo/ $10^{12}$  nucleosides/Gy at 5.0 kGy. Again, measurements of 8-oxo-dGuo after irradiation have previously been measured mainly using eukaryotic cells<sup>25,31–33</sup> and with doses lower than 500 Gy. In the THP1 malignant monocyte cell line, HPLC-MS/MS analysis indicated a damage yield reaching 20 8-oxo-dGuo/ $10^9$  nucleosides/Gy ( $\sim 200$  to 300 8-oxo-dGuo/cell/Gy).<sup>31</sup> Kish et al.<sup>28</sup> analyzed the oxidized base, 8-oxoG, after exposure of the archaeon *H. salinarum* at 0, 2.5, 5.0, and 7.5 kGy. A plateau was reached at 2.5 kGy, for which the levels were 2.1 8-oxoG/ $10^6$  bases and 3.1 8-oxoG/ $10^6$  bases at 0 and 7.5 kGy, respectively (<sup>60</sup>Co gamma source), corresponding to less than 1 lesion/ $10^{12}$  bases/Gy. Therefore,

this archaeon also showed yields of oxidized guanine formation far below those observed in human cells. These very low yields may be explained by a cellular redox environment and low O<sub>2</sub> levels that prevent the formation of oxidative DNA damage, or by detoxification systems.<sup>4</sup> In the same halophilic organism, Robinson et al.<sup>34</sup> observed an increase of FapyGua levels at 2.5 kGy but no significant progression of 8-oxoG.

Apart from a potential role of DNA repair enzymes, low levels of oxidative DNA damage observed in *T. gammatolerans* after irradiation may be linked to mechanisms such as highly constitutively expressed detoxification systems. For example, the genome of this archaeon is containing sequences related to proteins known to decrease oxidative stress such as a thioredoxin reductase (TGAM\_0180), a glutaredoxin-like protein (TGAM\_1302), and 2 peroxiredoxins (TGAM\_1253 and TGAM\_1220).<sup>2,4</sup>

*T. gammatolerans* cells exposed to 5.0 kGy radiation showed a significant decrease of 8-oxo-dGuo after 1 h recovery in culture medium, whereas this phenomenon was not significant at the lower levels of oxidative DNA damage observed at 2.5 kGy. Studies performed with *H. salinarum* indicated a decrease of FapyGua, FapyAde, and 8-oxoG rates after a 2.5 kGy irradiation and 2 h recovery compared with nonexposed organisms (rates after 1 h recovery were not measured).<sup>28</sup> This decrease of 8-oxo-dGuo 1 h after the end of irradiation may contribute to the resistance of *T. gammatolerans*. It may be explained by DNA repair systems or other factors limiting the extent of DNA damage.

**Cyclonucleoside Formation in *T. gammatolerans* DNA Is Questionable.** Cyclonucleosides (in this study, cyclopurines) are classified as complex oxidative DNA lesions presenting, in the same nucleotide, a covalent bond between the C8 of the base and the C5' of the 2-deoxyribose. *In vitro* studies show that these bulky lesions are mutagenic<sup>35</sup> and interfere with replication and transcription.<sup>36–38</sup> Contrary to small oxidative DNA damage such as oxidized bases, it also appears that cyclopurines are repaired by the nucleotide excision repair system and not by BER enzymes. In parallel to these mechanistic results, measurements of cyclopurine rates in DNA extracted from eukaryotic cultured cells and tissues have been performed by a limited number of research teams. In spite of similar methods, i.e., isotope dilution techniques in combination with HPLC coupled to mass spectrometry, discrepancies can be noted among the concentrations reported in the literature. In THP1 human monocytes, Belmadoui et al.<sup>9</sup> did not detect 5'R and 5'S diastereomers of 5',8-cyclodAdo above the detection limit of the method, which was 0.1 lesion per  $10^9$  nucleosides. In rodent tissues, the team of Wang measured basal levels of 5'R and 5'S diastereomers of 5',8-cyclodAdo and 5',8-cyclodGuo superior to 0.1 lesion per  $10^6$  nucleosides.<sup>39–41</sup> In the work reported here, we did not show the presence of detectable cyclopurines in *T. gammatolerans* cells in any of the conditions tested. Indeed, even exposure to 5.0 kGy radiation did not lead to a signal above our limit of detection, estimated at 1 lesion/ $10^9$  nucleosides for this experiment (the limit of detection is dependent on the quantity of DNA available for the analysis). In our case, another favorable factor for the detection of cyclonucleosides should be the anaerobic growth conditions of *T. gammatolerans*. Cyclonucleosides are indeed more prone to formation at low O<sub>2</sub> concentrations.<sup>9</sup> Taken together, these data indicate that cyclonucleoside formation in living cells, *T. gammatolerans* in our case, may occur at very low levels, if at all.

			Q31
Pa-AGOG	1	MAAESQLKRVIE <sup>!</sup> TLRRLGIEV <sup>!</sup> LKLERR	D <sup>!</sup> PQYRAVCN <sup>!</sup> VWKR HGETV
TGAM_1653	1	MTLDRFV RIKYREDNEKVNRLVE <sup>!</sup> ILRELGLDCARTIE <sup>!</sup> EKV <sup>!</sup> DLQFDALRNLRLENLKDDEL	
TGAM_1277	1	MSLDGFTFEESWDEKRRRAEKIV <sup>!</sup> EILMRTHPREKLLIG	D <sup>!</sup> PYRTLHICIIISQMRDE <sup>!</sup> V
Consensus		m.ld.f.....e.....r. <sup>!</sup> EiLrrlg..e.l.ie...Dpq..a.n.....#ev	
Pa-AGOG	47	GSRLAMLNALISYRLTGKGE <sup>!</sup> HW <sup>!</sup> EYFGKYFSQLEVIDLCRDFLKYIETS <sup>!</sup> PFLKIV <sup>!</sup> EARK	
TGAM_1653	60	PIKLVIANALVSYQLSGKGE <sup>!</sup> W <sup>!</sup> W <sup>!</sup> EF SRYFS <sup>!</sup> ENPPEDIVEAYS <sup>!</sup> SFLPNSK <sup>!</sup> TNRRLV <sup>!</sup> AGKL	
TGAM_1277	58	TYRV	W <sup>!</sup> EELFKKYGD <sup>!</sup> IB <sup>!</sup> TIANTPVDEMREFLRKRGVGLWKT <sup>!</sup> KG
Consensus		..rl...nal.sy.l.gkge..Weef.ky% <sup>!</sup> s# <sup>!</sup> .e.id.....sk....lv..k.	
Pa-AGOG	107	KRALKA <sup>!</sup> CDYV <sup>!</sup> PNLE	DLGLTLRQLSHIVGARRE <sup>!</sup> QK <sup>!</sup> LVFTIK <sup>!</sup> ILN <sup>!</sup> YAMCS
TGAM_1653	120	KRIERVEPPLSPLSISEIRDY <sup>!</sup> YFNGMERLRDE <sup>!</sup> LARVMKAK <sup>!</sup> RS <sup>!</sup> AKTIVFA <sup>!</sup> VMFGYAGRIA	
TGAM_1277	100	EWIVKASRIILEK	YGGKVPDDIKE <sup>!</sup> LMKLP <sup>!</sup> GIGRKCANTVLA <sup>!</sup> YGFGRQAI <sup>!</sup> PVD
Consensus		kri.ka.....l.....y.....#L....ga.R..ktiVfa.k...yA....	
Pa-AGOG	157	RGVNRVLPFDIPIV <sup>!</sup> DYRVARL <sup>!</sup> TWCAGLIDFPPEALRRYEA <sup>!</sup> VQKIWD <sup>!</sup> AVARET <sup>!</sup> GIPPLH	
TGAM_1653	180	FSAFVYPMAIEIPD <sup>!</sup> DVRINAYT	KRFTSEPPVS
TGAM_1277	152	THVNRISKRLGLAPPRV <sup>!</sup> PEKVE	EYLMELI <sup>!</sup> PK <sup>!</sup> EWIYNHAMVDHGRSICR <sup>!</sup> IRPKCE
Consensus		..vnr..p..i.i.iP.dvr...t...l...pP.e.....wgr.a.etgiPplh	
Pa-AGOG	217	IDTL <sup>!</sup> LW	L <sup>!</sup> AGRAVLYGENLHGVPKEVIALFQWRGGCRPPSE
TGAM_1653	228	IDSI <sup>!</sup> LWPVLGGKGEVLRRLK <sup>!</sup> KHCGEKAERILELRDL	
TGAM_1277	210	SCPLKELCPYAKGLVR <sup>!</sup> DEIK	
Consensus		.d.llw..l.gkv.v.e.k.....r.....	

**Figure 9.** Sequence alignment of *Pyrobaculum aerophilum* 8-oxoG DNA glycosylase (Pa-AGOG), TGAM\_1653, and TGAM\_1277. Identical residues are in red, and residues shared by two proteins are in blue. Residues playing an important role in catalytic activity or 8-oxo-dGuo recognition in Pa-AGOG (Q31, K140, K147, D172, D218, and W222) are indicated in green. The alignment was performed using the MultAlin tool<sup>62</sup> followed by manual editing.

**TGAM\_1653 and TGAM\_1277 Are Slightly Upregulated after Heavy Radiation Exposure.** Data extracted from proteomic results indicated the presence at low levels of BER enzymes TGAM\_1653 and TGAM\_1277 in nonirradiated and irradiated cells. No difference between these two conditions was noted in terms of protein abundance, but the sensitivity of the MS shotgun approach is not sufficient to detect moderate variations. The expression of the corresponding genes was confirmed by RT-PCR. For TGAM\_1277, the main difference appeared 2 h after a 5.0 kGy irradiation, with an increase of the relative expression from 0.03 (T0) to 0.09. TGAM\_1653 basal mRNA levels were higher than those of TGAM\_1277, but the regulation of the transcription of this gene was less sensitive to irradiation. The expression of BER enzymes before exposure to ionizing radiation is not surprising since extremophiles need constitutive protective mechanisms to deal with their harsh growth conditions. The DNA damage analysis reported here shows the presence of an elevated amount of oxidized bases in basal conditions that may require a sufficient amount of DNA repair enzymes. The weak or absent upregulation of several DNA repair genes after exposure to genotoxic stress has already been observed in other archaea. *Pyrococcus furiosus* subjected to 2.5 kGy <sup>60</sup>Co  $\gamma$ -rays did not show changes in mRNA levels for BER glycosylases but presented a moderate upregulation of *radA* (DNA repair by homologous recombination) and of a DNA repair gene cluster possibly involved in translesion synthesis.<sup>42</sup> UV irradiation of hyperthermophilic *Sulfolobus* archaea did not lead to overexpression of DNA repair genes, including BER genes.<sup>43</sup>

It is also recognized that a majority of DNA repair factors are constitutively expressed in eukaryotic cells.<sup>44</sup> At the protein level, the expression of BER factors is not massively induced, even after significant exposure to genotoxic agents.<sup>45</sup> We also observed that 8-oxo-dGuo produced after  $\gamma$ -ray exposure are repaired within the first hour of recovery, whereas maximum levels of TGAM\_1277 and TGAM\_1653 transcripts were measured at 2 h. We can suggest that newly synthesized enzymes are produced (i) to repair other types of more

persistent oxidative DNA damage or (ii) to reconstitute the pool of constitutive enzymes that has probably been, at least partly, altered by irradiation. This degradation of BER factors by  $\gamma$ -rays has already been shown to occur in human cells<sup>46</sup> at much lower doses (up to 60 Gy) compared to those in the present study. Finally, it is worth mentioning that our observations are mainly based on mRNA levels. It would be interesting to complement them with further data related to proteins, as well as their post-translational modifications, which are known to play an important role in the regulation of BER in eukaryotes.<sup>47</sup>

**TGAM\_1653 Excises 8-OxoG, TGAM\_1277 Excises 8-OxoG and Hydantoin Lesions, and Both Possess  $\beta$ -Elimination Activity.** According to our study, TGAM\_1277 and TGAM\_1653 are bifunctional BER enzymes: they are able (i) to recognize and remove oxidized bases by cleaving the *N*-glycosyl bond between the deoxyribose and the damaged base, and (ii) to cleave the resulting abasic site leading to a single-strand break (lyase activity). More precisely, we demonstrated that both enzymes cleave the abasic site via a  $\beta$ -elimination process resulting in a 3'-phospho- $\alpha,\beta$ -unsaturated aldehyde and in a 5'-phosphate extremity (Figure 6).

TGAM\_1277 was annotated as a putative type III endonuclease.<sup>2</sup> Indeed, we have shown here that this enzyme shares the common features of the bacterial BER enzyme, i.e., bifunctionality, a  $\beta$ -elimination process and the capacity to repair hydantoin lesions (5-OHdHyd and 5-OH-5-Me-dHyd).<sup>22,48</sup> By contrast with bacterial EndoIII, we found that TGAM\_1277 is also able to cleave 8-oxo-dGuo, particularly when mispaired with T or G. This variety of substrates is also found for the *E. coli* enzyme Fpg, which presents a  $\beta,\delta$ -elimination mechanism.

When compared with TGAM\_1277, TGAM\_1653 shows a more restrictive panel of substrates as it does not cleave oxidized pyrimidines and is not as efficient as TGAM\_1277 in cleaving 8-oxo-dGuo when mispaired, particularly to A and G. This profile of substrates is close to that noted in other studies

for the human BER enzyme, hOGG1 (human 8-oxoG DNA Glycosylase I).<sup>49,50</sup>

As for TGAM\_1277 and TGAM\_1653, the 8-oxoG DNA glycosylase from the sulfate-reducing archaeon *Archeoglobus fulgidus* (Afogg) presents a bifunctional and  $\beta$ -elimination mechanism.<sup>51</sup> This protein is able to repair 8-oxo-dGuo when associated with C and G, and to a lesser extent when mispaired with T and A. *Pyrobaculum aerophilum* 8-oxoG DNA glycosylase (Pa-AGOG), a formative member of a new DNA glycosylase family, also demonstrated a bifunctional and  $\beta$ -elimination process.<sup>10</sup> Structural studies indicated that this enzyme has an HhH-GPD motif (helix-hairpin-helix associated with a glycine-proline-rich sequence, followed by a conserved aspartate) that is significantly different in terms of amino acid sequence compared to that of hOGG1 but similar in terms of tridimensional structure.

Alignment of the Pa-AGOG, TGAM\_1277, and TGAM\_1653 polypeptides is shown in Figure 9. Pa-AGOG and TGAM\_1653 display only 25% sequence identity and 43% similarity, but *P. aerophilum* and *T. gammatolerans* belong to two separate phyla of Archaea (Crenarcheota vs Euryarcheota). However, residues playing an important role for catalytic activity (K140, D172) or substrate recognition (Q31, K147, D218, and W222) in Pa-AGOG<sup>10,52</sup> are conserved in TGAM\_1653 and not in TGAM\_1277 (Figure 9). This suggests that Pa-AGOG and TGAM\_1653 may belong to the same glycosylase family, even if some discrepancies are revealed. Pa-AGOG is able to repair 8-oxo-dGuo when paired with C and A but presented a stronger efficiency for 8-oxoG:G mismatches.<sup>52</sup> We showed here that this strong capacity to cleave 8-oxoG:G pairs was also shared by TGAM\_1277, TGAM\_1653 and Afogg. It could be interesting for hyperthermophilic organisms to cleave 8-oxoG:G lesions generated by the incorporation of free 8-oxodGMP opposite to a G, this phenomenon probably being favored by high temperatures.<sup>10,53</sup> Neither Afogg nor Pa-AGOG was tested for its capacity to repair damaged pyrimidines, making the comparison with *T. gammatolerans* enzymes difficult to extend further. We also tested cyclonucleosides as potential substrates: TGAM\_1277 and TGAM\_1653 did not show cleavage activity. Previously published experiments, based on eukaryote whole-cell extracts or purified enzymes, have already demonstrated that BER does not deal with these complex oxidative DNA lesions.<sup>54–57</sup>

In order to obtain a comprehensive picture of BER in *T. gammatolerans*, complementary experiments have to be performed on other putative BER factors highlighted by our previous genomic and proteomic screenings.<sup>2</sup> At least seven candidates, annotated as “uracil-DNA glycosylase”, “base excision DNA repair protein, HhH-GPD superfamily”, “AP endonuclease family 2”, or “endonuclease V”, have been identified. Study of these archaeal enzymes can lead to the discovery of original DNA repair activities, as revealed by the recent characterization of EndoQ found in *Pyrococcus furiosus* and *T. kodakarensis*.<sup>58</sup> This endonuclease is able to repair deaminated bases generated, for example, by ionizing radiation or elevated temperatures. Glycosylases and lyases are the very first enzymes involved in the BER system. It may also be interesting to study other BER proteins involved in the later stages of the pathway. For example, it would be relevant to focus attention on BER factors processing single strand breaks as these latter lesions are also generated by ionizing radiation.<sup>47</sup> Finally, a further characterization of enzymatic activities including parameters such as thermostability or influence of

pH, would be interesting, particularly for a potential use of these proteins as new tools for research and applied biotechnologies laboratories.<sup>59</sup>

## CONCLUSIONS

This work established that the basal level of the oxidative DNA damage, 8-oxo-dGuo, in *T. gammatolerans* is higher than that reported for mammalian cells. As expected, this level of damage increased with high doses of ionizing radiation. Remarkably, the yield of lesions generated was much lower than those observed in human cell lines. One hour postirradiation, the rate of oxidative DNA damage was the same as the control background, indicating that the repair process is rather rapid. TGAM\_1277 and TGAM\_1653 BER enzymes were slightly upregulated after heavy radiation exposure. As shown here, these enzymes are able to initiate DNA repair of oxidized pyrimidines (TGAM\_1277) and guanines (TGAM\_1277 and TGAM\_1653). Therefore, *T. gammatolerans* is at any time prepared to counteract DNA oxidation with this protein arsenal. While the search for cyclonucleosides showed no significant levels above technical background, other original DNA lesions such as clusters of damage<sup>33,60</sup> would be interesting to identify in radiotolerant organisms such as *T. gammatolerans*. Regarding DNA repair proteins such as TGAM\_1277 and TGAM\_1653, structural studies with the enzymes alone and with their DNA substrates would provide additional data about recognition and cleaving mechanisms.

Further molecular studies on proteins from *T. gammatolerans*, one of the most radiotolerant organisms currently known, should bring valuable information on DNA repair in archaea. Moreover, deciphering the mechanisms explaining its extraordinary resistance to extreme conditions may have potential applications in biotechnology.<sup>61</sup>

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00128.

List of oligonucleotides used for quantitative real-time RT-PCR and cloning and expression of TGAM\_1277 and TGAM\_1653-primer pairs used to amplify coding sequences from total genomic DNA (PDF)

## AUTHOR INFORMATION

### Corresponding Author

\*CEA Grenoble, INAC, Service Systèmes Moléculaires et nanoMatériaux pour l'Energie et la Santé (UMR E\_3 CEA/ Université Grenoble Alpes), 17 rue des Martyrs, 38054 Grenoble cedex 09, France. Tel: +33-0-4-38-78-56-01. E-mail: jean.breton@cea.fr.

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## ■ ABBREVIATIONS

5',8-cyclodAdo, 5',8-cyclo-2'-deoxyadenosine; 5',8-cyclodGuo, 5',8-cyclo-2'-deoxyguanosine; 5-OH-5-Me-dHyd, 1-[2-deoxy- $\beta$ -D-erythro-pentofuranosyl]-5-hydroxy-5-methylhydantoin; 5-OH-dHyd, 1-[2-deoxy- $\beta$ -D-erythro-pentofuranosyl]-5-hydroxyhydantoin; 8-oxo-dGuo, 8-oxo-2'-deoxyguanosine; 8-oxoG, 8-oxo-7,8-dihydroguanine; Afogg, 8-oxoG DNA glycosylase from the sulfate-reducing archaeon *Archeoglobus fulgidus*; BER, base excision repair; CDS, coding DNA sequence; Endo III, type III endonuclease; ESI, electrospray ionization; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; Fpg, formamidopyridine DNA N-glycosylase; FPLC, fast protein liquid chromatography; HhH-GPD motif, helix-hairpin-helix associated with a glycine-proline-rich sequence, followed by a conserved aspartate; hOGG1, human 8-oxoG DNA glycosylase 1; HPLC-MS/MS, HPLC coupled to tandem mass spectrometry; kGy, kiloGray; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; Mb, megabase; NSAF, normalized spectral abundance factor; ODN, oligodeoxynucleotide; Pa-AGOG, *Pyrobaculum aerophilum* 8-oxoG DNA glycosylase; radA, DNA repair by homologous recombination; ROS, reactive oxygen species; rpoB, DNA-directed RNA polymerase subunit B; RT-PCR, reverse transcription PCR; Tbp, TATA box-binding protein

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