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ORIGINAL PAPER

# Recovery of ionizing-radiation damage after high doses of gamma ray in the hyperthermophilic archaeon *Thermococcus gammatolerans*

**Angels Tapias · Christophe Leplat · Fabrice Confalonieri**

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**Abstract** The recently discovered hyperthermophilic and radioresistant archaeon *Thermococcus gammatolerans* is of great interest to compare and contrast the impact of its physiology on radioresistance and its ability to repair damaged chromosomes after exposure to gamma irradiation with radioresistant bacteria. We showed that, in contrast to other organisms, cell survival was not modified by the cellular growth phase under optimal growth conditions but nutrient-limited conditions did affect the *T. gammatolerans* radioresistance. We determined the first kinetics of damaged DNA recovery in an archaeon after exposure to massive doses of gamma irradiation and compared the efficiency of chromosomal DNA repair according to the cellular growth phase, nutrient availability and culture conditions. Chromosomal DNA repair kinetics showed that stationary phase cells reconstitute disrupted chromosomes more rapidly than exponential phase cells. Our data also revealed that this radioresistant archaeon was proficient to reconstitute shattered chromosomes either slowly or rapidly without any loss of viability. These results suggest that rapid DNA repair is not required for the extreme radioresistance of *T. gammatolerans*.

Angels Tapias and Christophe Leplat contributed equally to this work.

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A. Tapias · C. Leplat · F. Confalonieri (✉)
Laboratoire de Génomique des Archaea,
Université Paris-Sud 11 CNRS UMR 8621,
Institut de Génétique et Microbiologie, Orsay, France
e-mail: fabrice.confalonieri@u-psud.fr

*Present Address:*
A. Tapias
Departamento de Biotecnología Microbiana,
Centro Nacional de Biotecnología, CNB, CSIC, Madrid, Spain

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## Introduction

Prokaryotes present a wide-ranging tolerance to $\gamma$-ray irradiation. The decimal reducing dose $D_{10}$ values for *Escherichia coli*, *Pseudomonas putida* and *Staphylococcus aureus* are 700, 250 and 120 Gy, respectively (Daly et al. 2004; Dion et al. 1994), while several *Deinococcaceae* among others bacterial species withstand $\gamma$-ray doses of several thousands Grays (de Groot et al. 2005; Makarova et al. 2007; Minton 1994). *E. coli* cells are killed with only a few DSBs per chromosome induced by ionizing radiation (Gerard et al. 2001; Krasin and Hutchinson 1977) whereas *Deinococcus radiodurans* is able to handle hundreds of DSBs without any loss of viability (Minton 1994). This bacterium encodes extremely efficient DNA repair systems that enable it to survive at DNA damage doses lethal for other living organisms (Bentchikou et al. 2007; Zahradka et al. 2006). However, even though DNA repair pathways play an important role in $\gamma$-ray resistance, other bacterial cellular features could promote DNA repair, minimize DNA damage, or protect cellular structures from the effects of $\gamma$-radiation, and consequently enhance cell survival after irradiation (Battista 1997; Daly et al. 2004, 2007; Minsky 2003; Zimmerman and Battista 2005).

Among prokaryotes, the radioresistance phenotype is not constrained to bacteria but also encompasses members of the Archaea that represent the third kingdom of life (Woese et al. 1990). Previous studies have shown that *Desulfurococcus amyloliticus*, several *Pyrococcus* and *Thermococcus* species, as well as the halophilic archaeon *Halobacterium* sp, are also radioresistant organisms, even

though they survive at doses lower than these found for several radioresistant bacteria (DiRuggiero et al. 1997; Gerard et al. 2001; Jolivet et al. 2004; Kottemann et al. 2005). *Pyrococcus* species can endure 2,000–3,000 Gy without loss of viability and several *Thermococcus* species can withstand higher doses. *Halobacterium* sp exhibits a 1% survival rate after an exposure to 6,000 Gy. An exposure to 2,000–3,000 Gy also generates DSBs in archaeal genomes that appear to be rapidly repaired (DiRuggiero et al. 1997; Jolivet et al. 2003b) but no data is available on their ability to repair the chromosome after exposure to very high doses of irradiation. Therefore, the only repair kinetics reported after irradiation under such conditions have been performed for bacterial species, using *Deinococcaceae* species as model organisms. Moreover, the physiological impact on archaeal radioresistance is less well documented than for bacteria. The growth phase influences *Halobacterium* radioresistance (Kottemann et al. 2005), and in contrast to radioresistant bacteria, exponentially growing cells are more resistant to gamma ray than stationary phase cells. However, this behavior has not been reported for any other archaeon, and no data is available on modulation of archaeal radioresistance profiles by growth medium composition or conditions of culture for hyperthermophilic species.

The radioresistant archaeon, *Thermococcus gammatolerans*, was recently discovered (Jolivet et al. 2003a) and belongs to the order of *Thermococcales*. *T. gammatolerans* was isolated after an exposure to a gamma irradiation dose of 30 KGy from an enriched culture of microorganisms collected at the Guyamas basin. As its name suggests, this organism can withstand a dose of 3,000 Gy without apparent lethality and an exposure to higher doses only slightly reduces its viability. These data indicate that *T. gammatolerans* is the most radioresistant archaeon isolated thus far, offering the chance to determine how archaeal species recover from extensive DNA damage after exposure to a massive radiation dose and under various conditions.

In the present work, we have evaluated the radioresistance of *T. gammatolerans* according to energy requirements and growth phase. In contrast to several organisms, growth phase does not influence radioresistance under optimal growth conditions, but comparative chromosomal DNA repair kinetics indicates that *T. gammatolerans* stationary phase cells are able to reconstitute the shattered chromosome faster than exponentially growing cells. Reconstitution of fragmented chromosomal DNA after irradiation at high doses is rapid, though it requires longer times than those reported for the model organism *D. radiodurans.*

Interestingly, our results also show that, depending on experimental conditions, *T. gammatolerans* cells are able to repair damaged chromosomes quickly or slowly with the same efficiency. These data suggest that rapid DNA repair is not a prerequisite for extreme radioresistance.

## Materials and methods

### Strains, media and growth conditions

*Thermococcus gammatolerans* was kindly provided by D. Prieur. The strain was grown in serum bottles or in Hungate tubes, under anaerobic conditions at 85°C either in complex organic medium (VSM) or in mineral medium (ASW) supplemented by a solution of amino acids, vitamins and sulfur. The complex organic medium VSM is composed of 20 g/l NaCl, 0.25 g/l KCl, 0.05 g/l NaBr, 0.02 g/l boric acid, 0.01 g/l $SrCl_2.6H_2O$, 0.5 g/l trisodium citrate, 3 g/l Pipes, 1 g/l Yeast extract, 4 g/l Bactotryptone, 5 ml $MgSO_4$ 20%, 1 ml $CaCl_2$ 5%, 1 ml $KH_2PO_4$ 5%. The pH was adjusted to 6.8 by addition of NaOH. The ASW-AA was prepared as described in (Sato et al. 2003). Both media were sterilized by autoclaving (105°C, 20 min) and transferred into individual serum bottles or Hungate tubes. Inorganic sulfur S° (sulfur flowers, Fischer Scientific) was added to a final concentration of 2 g/l. Air contained in the bottle or tube was first removed using a vacuum and then replaced by $N_2$ (Air Liquide, France). To reduce the oxygen dissolved in the medium, $Na_2S$-$9H_2O$ at a 0.1% final concentration was added. The media were checked until the color of resazurin sodium salt (1 mg/l) became clear.

Typically, VSM-S° cultures were performed after inoculation at a cellular density of $2 \times 10^5$ cells/ml into 25 ml of fresh medium in a 75 ml serum bottle. The exponential growth phase represents a cellular density of $2 \times 10^7$ cells/ml reached after 7 generations. Cellular division is asynchronous and at least 40% of the total population was in division. Stationary phase is characterized by an increase in cell density ($5 \times 10^8$–$10^9$ cells/ml reached after 13–14 generations) and cellular division events become sporadic. The late stationary phase concerns cells maintained at 85°C under agitation in the same medium for 30 h after stationary phase was reached. The ASW-AA-S° cultures were grown in a comparable way. The inoculations were performed at a cellular density of $10^6$ cells/ml and incubated at 85°C for 5 generations. The stationary phase is reached after 9–10 generations. The cell culture densities were followed by optical microscopy (Olympus BH-29) using a Thoma counting chamber (Microgravure Precis) and stopped by rapid cooling at the appropriate growth phase and cell density.

### Cell survival after gamma irradiation

VSM and ASW-AA cultures supplemented with sulfur flowers were incubated on ice at indicated growth phases and cellular densities. All further manipulations were carried out in an anaerobic chamber on ice, except centrifugations which were performed outside. Cultures were filtred to eliminate residual sulfur. Cells were harvested (2,000*g*, 20 min at 4°C, Jouan GR412) and resuspended the corresponding fresh reduced medium to concentrate them ten-fold. Equal aliquots of concentrated cells of 0.8 ml were then placed into Hungate tubes and irradiated on ice at a rate of 42.5 Gy/min using a $^{137}$Cs $\gamma$-ray source (IBL637 Cis Bio International, Institut Curie, Orsay). The same number of non-irradiated control cells were stored on ice during irradiation and processed at the end of the manipulation. Following irradiation and for each dose, serial ten -fold dilutions were prepared in the reduced fresh medium (either VSM or ASW-AA) until a cellular density of 0.033 cells/ml and 0.3 ml or 1 ml of these dilutions were used to inoculate Hungate tubes containing 2.7 ml of fresh medium or 24 ml in serum bottles, respectively.

The cultures in bottles and tubes were incubated at 85°C for a maximum of 9 days. The inocula were checked every 24 h for the presence or absence of growth by optical microscopy using a Thoma counting chamber. Cell survival was evaluated according to the last positive dilution where cells were able to restore a high cellular density culture (>$10^7$ cells/ml) by comparison of dilutions of non-irradiated cells used as an internal reference. All dilutions were performed in duplicate and three biological replicates were included. The survival fractions were calculated by the most probable number method.

### Pulsed field gel electrophoresis

Cultures were prepared according to the method described in previous sections and pooled to assure a large number of cells (at least 3 × $10^{10}$ cells). Cells were then sampled in a fixed volume of 0.8 ml. Irradiated and non-irradiated control cells were then incubated at 85°C in the corresponding medium (ASW-AA-S° or VSM-S°) at a density of at least $10^7$ cells/ml. At regular post-irradiation incubation times (each hour), samples were taken to prepare DNA plugs as described in (Gerard et al. 2001) at a cellular density of at least $10^8$ cells/plug. Plugs were then washed in TE (10 mM Tris, 1 mM EDTA pH 8) containing 1 mM PMSF (phenylmethylsulphonyl fluoride) and stored at 4°C in TE solution.

Just before digestion, plugs were extensively washed in sterile water, incubated 1 h in the buffer of the restriction enzyme supplied by the manufacturer (New England Biolabs) and then digested with 40 units of *Swa*I enzyme in a volume of 100 µl per plug for 6 h at 30°C. The restriction enzyme was inactivated by incubation at 65°C for 20 min. Digested chromosomal DNA was analyzed on 1% agarose gels in 1× TBE (Euromedex) using a CHEF-MAPPER electrophoresis system (Bio-Rad) under the following conditions : 5.5 V/cm, 10°C, with a linear pulse of 40 s and a switch angle of 120° (−60° to +60°), for 30 h. For each $\gamma$-ray dose, chromosomal DNA repair kinetics were carried out in parallel. PFGE kinetics are representative of at least two independent cultures.

### Growth recovery without or after irradiation

Stationary and exponential growth phase cultures in ASW-AA-S° and VSM media were performed as previously described. The cells were filtered to eliminate sulfur and sampled in tubes. Cells were then irradiated at the appropriate dose and non-irradiated control cells were kept on ice for 1–3 h. Then they were inoculated in serum bottles or Hungate tubes at a minimal cellular density of $10^7$ cells/ml in the same reduced medium and sulfur was added at a concentration of 2 g/l. Generally, the inocula were checked every hour for the presence or absence of growth by optical microscopy using a Thoma counting chamber.

## Results

### Influence of the age of the culture on *T. gammatolerans* radioresistance

Among the parameters that could modulate radioresistance, the growth phase may be one of the relevant factors. To evaluate the radioresistance profile of this archaeon, we set up the following assay. Exponentially growing and stationary phase cells grown in sulfur supplemented rich-nutrient medium (VSM-S°) were harvested under anaerobic conditions and exposed to gamma irradiation as described in the Materials and Methods section. In our experiments, cells are generally exposed to three different $\gamma$-ray doses. A basic dose (2,500 Gy) and also a double and triple dose (5,000 and 7,500 Gy, respectively) that induce in *T. gammatolerans* detectable chromosomal DNA damage on Pulsed Field Gel Electrophoresis (PFGE) experiments (Fig. 2). After irradiation, serial dilutions were performed in the same fresh liquid medium and incubated at 85°C in the presence of elemental sulfur. The inocula were observed for the presence or absence of growth by optical microscopy using a Thoma counting chamber. Cell survival was evaluated according to the last positive dilution able to restore a high cellular density culture (>$10^7$ cells/ml) in comparison with dilutions of non-irradiated

cells used as a reference. For each dose, at least three independent replicates were performed, all dilutions were duplicated and the survival fractions were calculated by the most probable number technique.

As shown in Fig. 1 exponentially growing *T. gammatolerans* cells survives without any loss of viability until at least 5,000 Gy, with a slight decrease of survival observed at 7,500 Gy (0.5%). This behavior is consistent with previously reported results (Jolivet et al. 2003a) and represents a typical resistance profile of an exceptionally radioresistant organism. Moreover, we showed that *T. gammatolerans* radioresistance is not phase-growth dependant as no difference of survival was detected at doses of up to 7,500 Gy under optimal growth conditions (VSM-S°) (Fig. 1). On the contrary, late stationary phase cells that remained viable at 85°C under agitation in the same medium for 30 h after stationary phase was reached, are more sensitive to irradiation. No differences are observed at doses up to 5,000 Gy,

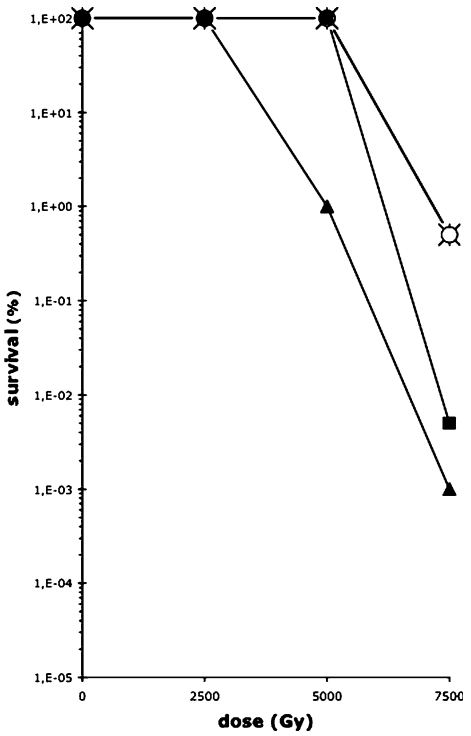


**Fig. 1** Effects of the age of the culture and of medium complexity on *T. gammatolerans* radioresistance. Survival of stationary and exponential growth phase cells cultured either under nutrient-rich conditions (VSM-S°) or nutrient-limiting conditions (ASW-AA-S°), after irradiation at the indicated $\gamma$-ray doses. Cells were irradiated in the same medium without sulfur and further incubated in the same fresh medium supplemented with sulfur flowers. Survival of VSM-S° exponentially growing cells (*asterisk*), VSM stationary phase cells (*open circle*), VSM late stationary phase cells (*filled square*), ASW-AA-S stationary cells (*filled triangle*) was determined by the MPN method. Values are the means of three experiments with duplicate cultures per experiment

but at a dose of 7,500 Gy, exponentially growing cells or early stationary phase cells appears 100-fold more radioresistant than late stationary phase cells. Consequently, the effects of $\gamma$-rays depend on the time that cells remained in this phase. Various hypotheses may explain these results: a late stationary phase culture contains cells engaged in a death program turned on prior to irradiation which may be enhanced at high $\gamma$-irradiation doses. The late stationary phase may also be composed of cells containing less antioxidant systems, or a fragile cell wall, thus increasing $\gamma$-ray sensitivity. We also cannot exclude the presence of an unknown radiation inducible virus located in the *T. gammatolerans* genome, which could induce cell lysis after exposure to very high doses of irradiation.

## The *T. gammatolerans* radioresistance is modulated by nutrient availability

*Thermococcus gammatolerans* was previously described as strictly anaerobic heterotrophic archaeon that requires yeast extract, tryptone and peptone to grow (Jolivet et al. 2003a). However, we were able to grow this organism in a mineral minimum medium supplemented with sulfur, vitamins, and a 20 amino acids solution (ASW-AA medium) (Sato et al. 2003).

Under these limiting conditions, culture parameters such as the lag period and doubling time change in comparison to those observed in VSM medium (Table 1). The generation time is longer (4–5 h) than in VSM-S°, and stationary phase is reached at a lower cellular density ($2 \times 10^8$ cells/ml). Lag periods (minimum 4 h) are always detected after harvesting the cells. This lag could be explained if *T. gammatolerans* cells need sulfur or sulfur-containing compounds that were accumulated in the medium during growth. These compounds would be then discarded after centrifugation, preventing a rapid metabolism restart. The carbon source available in the ASW-AA-S° medium is provided by a combination of the 20 amino acids. Therefore, *T. gammatolerans* must synthesize all other compounds required to grow from these amino acids, thus extending the generation time. Consequently, we wondered how such culture conditions could influence radioresistance.

The behavior of *T. gammatolerans* in minimal medium is different from that observed in VSM-S° medium. The stationary phase cells exhibit 100% viability until 2,500 Gy and drastically decreases to reach values of 1% survival at 5,000 Gy and 0.001% survival at 7,500 Gy (Fig. 1). When cells are irradiated in exponential growth phase, survival is maintained until a dose of 2,500 Gy, but further exposure to higher gamma irradiation doses results in an important cell mortality that is also observed if cells are not exposed to DNA damaging agents (data not shown). This suggests that a disturbance of an active metabolism generated after

**Table 1** Estimated time for *T. gammatolerans* non-irradiated cells to recover growth (in minutes or hours) after incubation on ice, according to the growth phase of culture (exponential or stationary) and to the medium (VSM-S° or ASW-AA-S°)

| | Medium: | VSM-S° medium | | | | ASW-AA-S° medium | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Growth phase: | Exponential | | Stationary | | Exponential | | Stationary | |
| | Time of incubation on ice | SB | H | SB | H | SB | H | SB | H |
| Time of growth recovery | 0 h | 0 h | 0 h | 0 h | 0 h | 4 h | 4 h | 19 h | 19 h |
| | 1 h | 0 h | 0 h | 0 h | 0 h | 10 h | 10 h | 19 h | 19 h |
| | 2 h | 0 h | 0 h | 1 h | 1 h | ND | ND | ND | ND |
| | 3 h | 30 min | 30 min | 4 h | 4 h | ND | ND | ND | ND |
| Generation time | | 1 h 30 min | 2 h | – | – | 4 h | 5 h | – | – |

Cells were inoculated in serum bottles (SB) or in Hungate tubes (H) at 85°C

The generation time is indicated in both cases

*ND* not determined

harvesting and a long incubation on ice is not well tolerated by *T. gammatolerans* cells under limiting nutrient conditions. Thus, we were unable to analyze cell viability under such condition. However, when either exponentially growing cells or stationary phase cells, pre-grown in ASW-AA-S°, were inoculated after irradiation in VSM-S° medium, the survival rates were fully restored and identical to those obtained when cells are grown in VSM-S° medium (data not shown). These data confirm that the effect of nutrient availability or energy after irradiation is important for the survival of *T. gammatolerans* cells.

## Comparative chromosomal DNA repair kinetics between stationary and exponentially growing cells in VSM-S° medium

Since the radioresistance of *T. gammatolerans* is comparable in VSM-S° medium either for stationary or exponentially growing cells, we wondered if their chromosomal DNA was identically damaged and if the kinetics of DNA repair were the same. We examined chromosomal DNA repair kinetics using PFGE. After irradiation, the cells were inoculated in fresh medium, and aliquots were placed in agarose plugs at regular time intervals. After cell lysis, genomic DNA was digested by the rare-cutting restriction enzyme *Swa*I. The *Swa*I digestion pattern of a non-irradiated genome consists of a DNA fragment of at least 550 kb and also several fragments with sizes less than 300 kb. In our conditions of migration all marker bands larger than 533 Kb fall outside the resolving power of the gels and co-migrate at the same position. Therefore, the *Swa*I fragment of 550 kb may be larger and since the *T. gammatolerans* genome is composed of a single circular chromosome of $\sim$2 Mb (unpublished results), the band represents at least one-fourth of the complete genome. The reappearance of the total digestion pattern with similar band intensities between irradiated DNA and non-irradiated cells is indicative of the time necessary for the cells to repair the shattered chromosomes.

After an exposure to a dose of 2,500 Gy (Fig. 2a) the 550 Kb *Swa*I genomic fragment disappears under all conditions, but not all the other moderate length fragments (100–250 Kb) indicating that the genome is not completely degraded. When irradiated cells are incubated at 85°C to allow DNA repair to take place, a maximum of 4 h is sufficient for exponentially growing cells (lane 15), and less than 3 h for stationary phase cells (lane 6), to completely reassemble their chromosomes.

As shown in Fig. 2b, an exposure to 5,000 Gy results in full degradation of the chromosomes (lanes 3 and 14). The restoration takes place after an incubation period of a maximum 5 h if cells were irradiated in stationary phase (lane 8) or a maximum of 6 h for exponentially growing cells (lane 20). A similar behavior was observed after exposure to 7,500 Gy (Fig. 2c). Complete chromosome repair is reached after a maximum of 10 h for stationary phase cells (lane 12) or 11 h (lane 28) for exponentially growing cells. Our results demonstrate that *T. gammatolerans* is able to efficiently repair extensive genome damage. Moreover, repair occurs after a period in which damaged chromosomal DNA is more extensively degraded (Fig. 2b, c). A comparable intermediate pattern of repair has been previously reported for the archaeon *P. abyssi* and for the bacterium *D. radiodurans* (Battista 1997; Bentchikou et al. 2007; Jolivet et al. 2003b; Zahradka et al. 2006). A slight difference (1–2 h) of chromosomal DNA repair kinetics was always observed between stationary phase and exponentially growing cells. The molecular size of fragments generated by $\gamma$-radiation appeared smaller in exponentially growing cells than in stationary phase cells (Fig. 2b, lane 3 vs. lane 14), but actively growing cells harbor replicating genome structures producing smaller

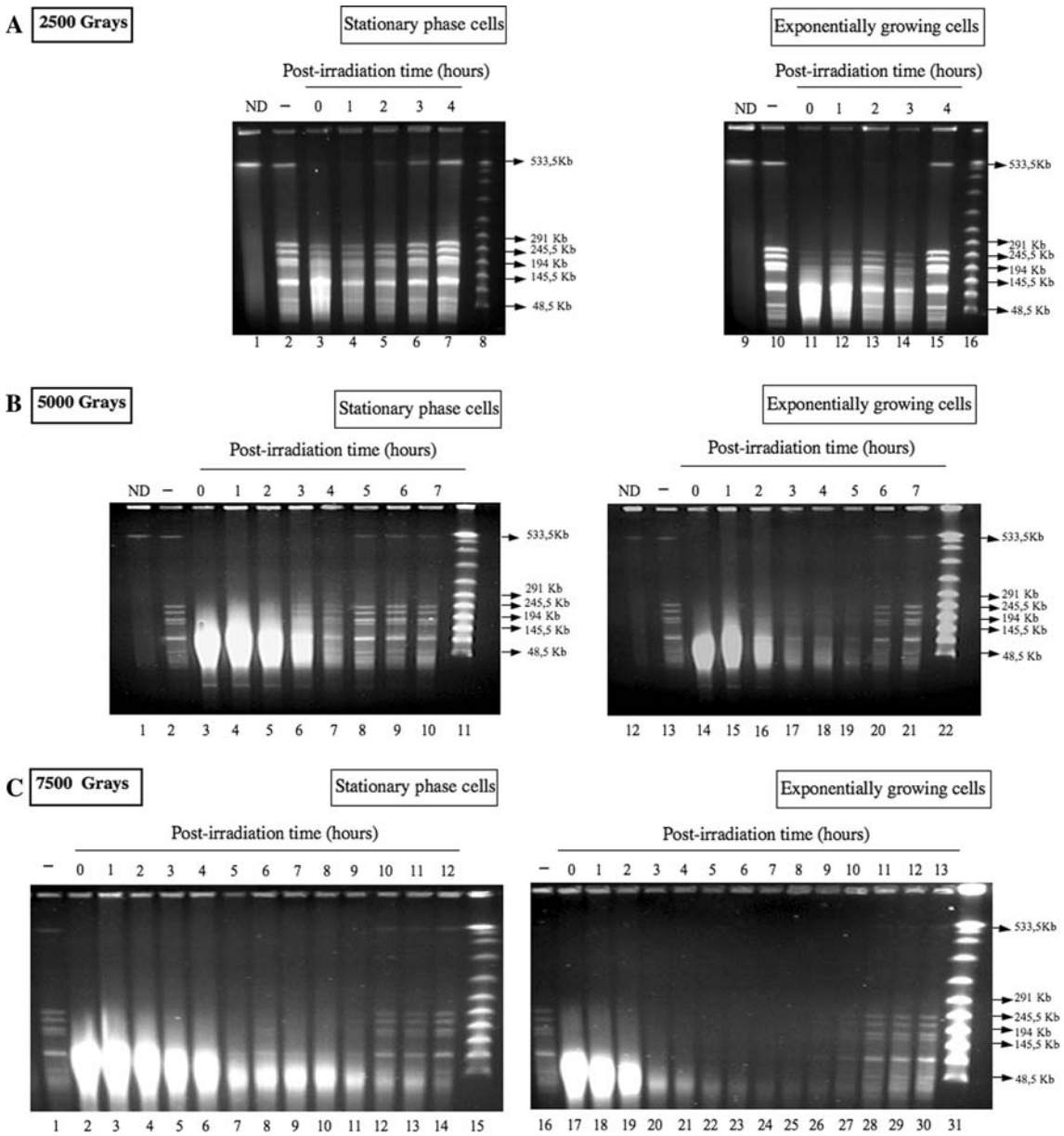


**Fig. 2** Time course analysis of *T. gammatolerans* chromosomal DNA repair after irradiation at increasing $\gamma$-ray doses. Chromosomal repair kinetics of *T. gammatolerans* VSM-S° exponentially growing cells (*right side* of the figure) or stationary phase cells (*left side*) following irradiation at doses of 2,500 Gy (**a**), 5,000 Gy (**b**) or 7,500 Gy (**c**). Time 0 represents an incubation of 5 min at 85°C. Genomic DNA was visualized after digestion by the rare cutting enzyme *Swa*I. ND symbol corresponds to non-digested genomic DNA of non-irradiated cells. *Straight line* refers to the digested genomic DNA extracted from non-irradiated cells. A DNA marker (lambda concatamers DNA, PFGE Marker, New England, Biolabs) was used as reference. The size of several bands are indicated by *arrows*

genomic double-stranded DNA fragments. Consequently, one cannot exclude that DNA repair kinetic variations are a consequence of less extensive DNA damage (less DSBs) in stationary phase cells. The viability of *T. gammatolerans* cells irradiated at 7,500 Gy is 0.5% but the DNA band intensities on PFGE gels (Fig. 2b, c) suggest that a large portion of the cells remained able to repair their chromosomal DNA.

## Chromosomal DNA repair kinetics under slow growth conditions

*Thermococcus gammatolerans* cells may be grown under anaerobic conditions in serum bottles or in Hungate tubes in the presence of elemental sulfur. The volume of the culture in Hungate tubes is limited to 3 ml. The generation time in tubes (120 min) is 33% longer than that observed in

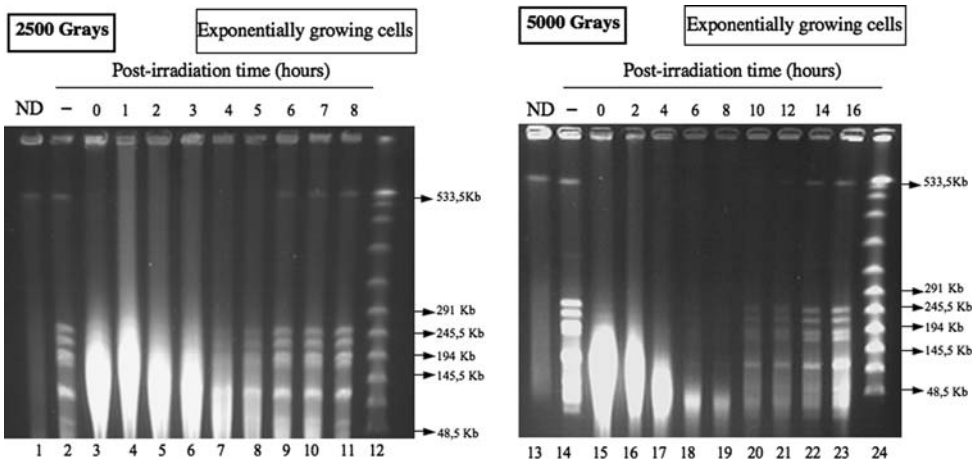


**Fig. 3** *T. gammatolerans* is able to repair the damaged chromosome with delayed kinetics. Chromosomal repair kinetics of *T. gammatolerans* VSM-S° exponentially growing cells following irradiation at a dose of 2,500 Gy (*left side* of the figure) or of 5,000 Gy (*right side*). After irradiation, cells were inoculated in Hungate tubes containing 3 ml of VSM-S° medium. Samples were taken at the indicated times (hours) to prepare agarose plugs. Time 0 represents an incubation of 5 min at 85°C. Genomic DNA was visualized after digestion by *Swa*I. ND symbol corresponds to Non-Digested genomic DNA of non-irradiated cells. *Straight line* refers to the digested genomic DNA extracted from non-irradiated cells. A DNA marker (lambda concatamers DNA, PFGE Marker, Biolabs) was used as a reference (*lane 12* and *lane 24*). The size of several bands are indicated by *arrows*

serum bottles (90 min) (Table 1). Therefore, we wondered if the survival rates could change according to the material used for the culture. Interestingly, the survival rates are the same in both conditions (data not shown), but the time required for recovering a high cell density culture after irradiation is longer in tubes. This can be partially explained by the longer generation time. However, radioresistant organisms are reputed to efficiently and rapidly repair all damage induced by ionizing radiation. This led us to ask if different growth conditions have an impact on the time required to repair shattered chromosomes. After an exposure to 2,500 Gy, reconstitution of shattered chromosomes from exponentially growing cells was observed within a maximum of 6 h (Fig. 3 lane 9) in contrast to the 4 h required when cells were grown in bottles. When cells were exposed to 5,000 Gy, a complete genome restoration occurred in Hungate tubes within 14–16 h whereas 6 h are required when cells are inoculated after irradiation in bottles. Our results suggest that repair kinetics were dependent of the conditions of culture used post-irradiation. Interestingly, since the survival rates are the same in both conditions (100%), a rapid genome restoration does not appear to be a prerequisite for the radioresistance of this organism.

## Chromosomal DNA repair kinetics under nutrient-limiting conditions

As shown in Fig. 1, nutrient conditions altered *T. gammatolerans* radioresistance profiles. We were therefore interested in determining the ability of cells to repair damaged chromosomes under such growth limiting conditions. As shown in Fig. 4, the chromosomal DNA of stationary phase cells is fully repaired within 6 h after irradiation at a dose of 2,500 Gy. In both nutrient-rich and nutrient-limiting conditions, the initial population represents surviving cells (100% survival, Fig. 2). At the same dose, 7–8 h are sufficient to repair the chromosomes of irradiated exponentially growing cells. Further assays, after a higher gamma radiation exposure, were not carried out since viability drops at a dose higher than 2,500 Gy. The genome reconstitution times that appeared longer in ASW-AA-S° medium may be a consequence of an extensive range of DNA damage to repair, since the pattern of fragments produced after irradiation in ASW-AA-S° medium are different than those observed in VSM-S° medium (Fig. 2a vs. Fig. 4). The bands of moderate length (100–250 Kb) observed in VSM-S° medium disappear in a broad smear which is comparable for both cellular growth phases. The repair of damaged exponentially growing cells genomic DNA remains longer than in stationary phase cells, as observed in VSM-S° medium and this extension is not strictly proportional to the generation time (Table 1).

## Recovery of growth without or after $\gamma$-irradiation

During irradiation, cells are kept on ice during the time necessary to produce $\gamma$-ray doses ranging from 2,500 to 7,500 Gy (typically 1–3 h). The cell density did not change but cell content may be affected. After irradiation, the cells were inoculated in fresh medium. We then estimated the time required to recover growth without irradiation or after

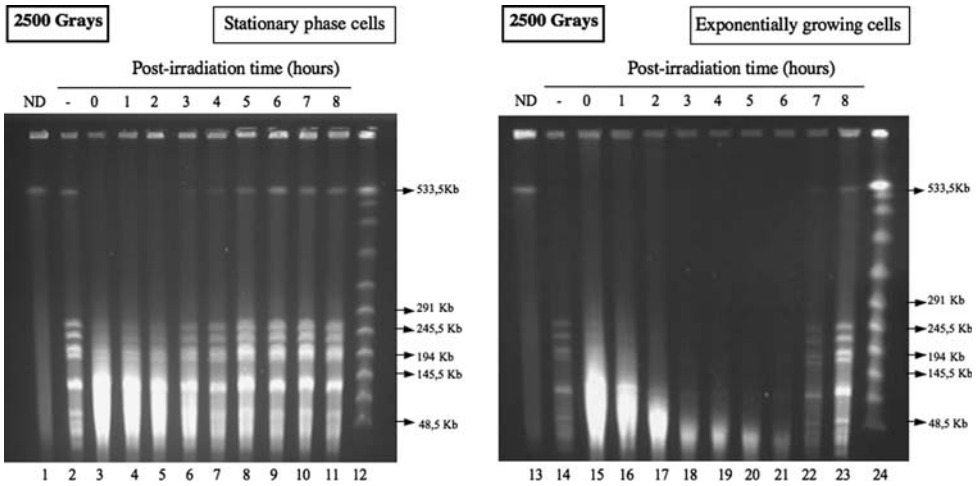


**Fig. 4** Time course analysis of *T. gammatolerans* genome reconstitution under nutrient-limiting conditions. Chromosomal repair kinetics of *T. gammatolerans* ASW-AA-S° exponentially growing cells (*right side* of the figure) or of stationary phase cells (*left side*), following irradiation at a dose of 2,500 Gy. After irradiation, cells were incubated at the indicated times (in hours). Time 0 represents an incubation of 5 min at 85°C. Agarose plugs were prepared and chromosomal DNA was digested by *Swa*I prior to analyses by PFGE. ND symbol corresponds to Non-Digested genomic DNA coming from non-irradiated cells. *Straight line* refers to the digested genomic DNA extracted from non-irradiated cells. A DNA marker (lambda concatamers DNA, PFGE Marker, Biolabs) was used as reference (*lane 12* and *lane 24*). The gels are representative of two independent cultures

gamma radiation at doses that produced full viability. As shown in Table 1, without irradiation, a lag was sometimes detected, which was not dependant on the material used (serum bottles versus Hungate tubes), but was dependent upon nutrient availability and the time the cells remained on ice after harvesting. The lag period sometimes increased for stationary phase cells and always under nutrient-limiting conditions. The recovery of growth for stationary phase cells grown in ASW-AA-S° medium is longer than for exponentially growing cells (19 h vs. 4–10 h) while their resistance to irradiation is higher (Fig. 1). The DNA repair kinetics also showed that stationary phase cells always more rapidly repair chromosomal DNA damage than exponential cells. This variation cannot be ascribed to a faster growth recovery, since the lag is longer for stationary phase cells (Table 1). If this parameter influenced the time necessary for *T. gammatolerans* to repair the damage, the recovery rates of stationary phase cells would have been significantly greater than for exponentially growing cells.

In serum bottles, cellular division restarts in a $\gamma$-ray dose-dependent manner, after complete cellular damage repair takes place (Fig. 5a, b). In Hungate tubes, the recovery of growth may be even longer since, at 5,000 Gy, it became visible after 28 h of incubation at 85°C (Fig. 5c) while the first cellular divisions were detected after 8–9 h in serum bottles. We did not evaluate the time necessary to recover growth after irradiation under nutrient-limiting conditions, our first results indicate that stationary phase cells irradiated at a dose of 2,500 Gy, require more than 40 h (data not shown), as non-irradiated cells restart an active growth phase after 19 h of incubation at 85°C. We found that the repair time of damaged chromosomal DNA is 1 h shorter for stationary phase cells than for exponential growing phase cells. This correlates with the variation of growth recovery observed in Fig. 5a, b. The generation time became identical to that of non-irradiated cells, indicating that the cells were viable. The exponentially growing cells appeared to restart after a transition of a minimum of 3 h. Gamma radiation induces extensive damage to all cellular components, not only chromosomal DNA. Since the lag displayed between complete chromosomal restoration and cell division is $\gamma$-ray dose-dependent, chromosomal DNA repair appears not to be the limiting event to control growth restart in *T. gammatolerans.*

## Discussion

We present here the first report of the impact of growth medium richness, culture age and culture conditions on an archaeon's radioresistance. Under optimal growth conditions, exponentially growing cells were able to endure $\gamma$-irradiation doses of up to 5,000 Gy without loss of viability, and supported doses of up to 7,500 Gy with 0.5% survival rate. These results are in apparent agreement with those presented by (Jolivet et al. 2003a). However, only one experimental condition (exponentially growing cells in rich-medium) was examined. Here, we showed that *T. gammatolerans* radioresistance was not growth phase-dependent. This result is in contrast to the behavior of *D. radiodurans*, which was found to be more radioresistant in stationary phase (Keller and Maxcy 1984; Minton 1994)

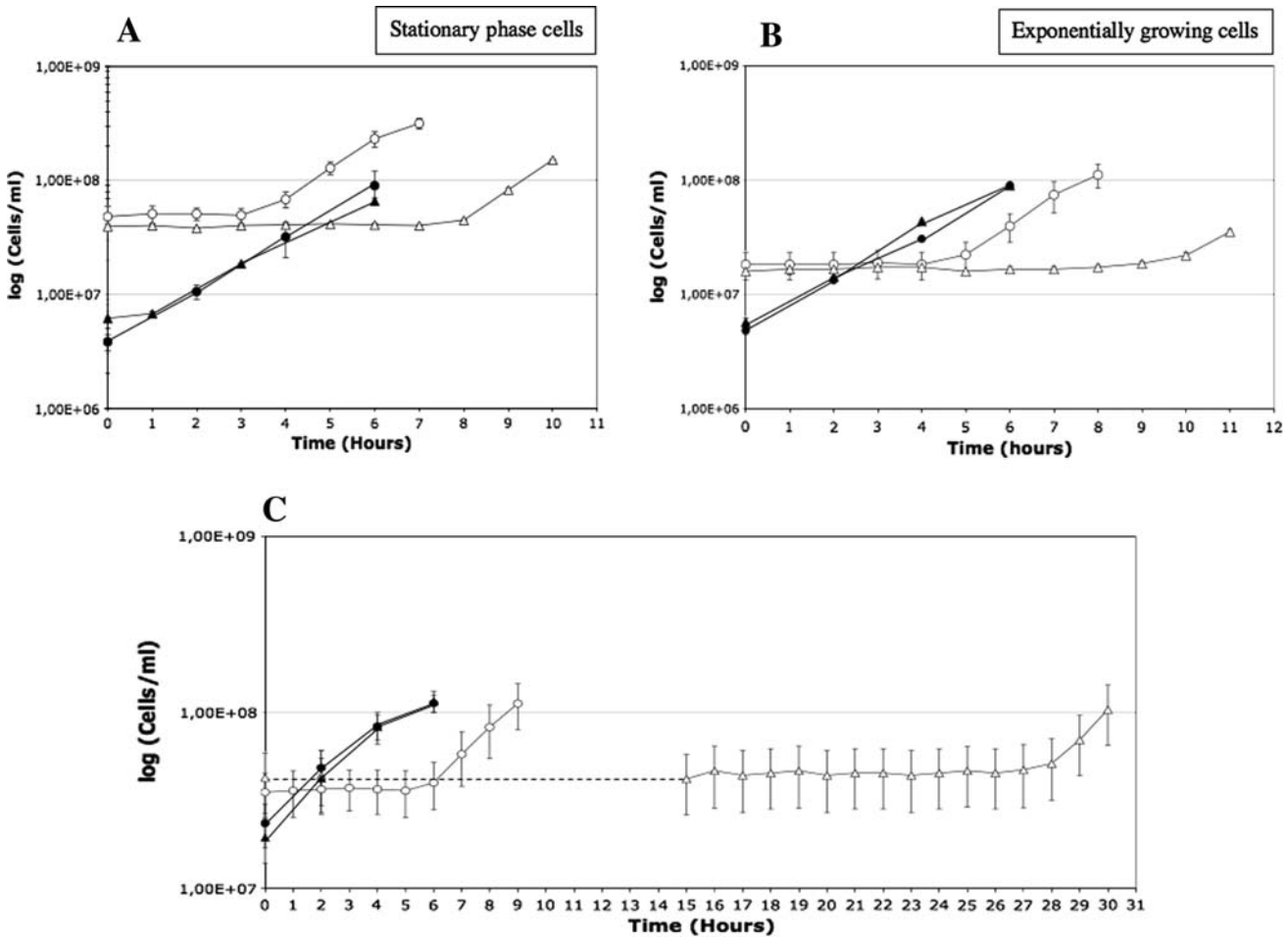


**Fig. 5** Recovery of growth in tubes and serum bottles at 85°C after irradiation. Recovery of growth after irradiation to a dose of 2,500 Gy (*open circle*), 5,000 Gy (*triangle*) of (**a**) *T. gammatolerans* stationary phase cells (**b**) exponentially growing cells, both in serum bottles containing VSM-S° medium or (**c**) exponentially growing cells inoculated in Hungate tubes containing VSM-S° medium. As controls, recovery of non-irradiated cells at appropriate growth phase kept on ice during 1 h (*filled circle*, time required to irradiate cells at a dose of 2,500 Gy) and 2 h (*filled square*, for a dose of 5,000 Gy) are indicated. The time is given in hours. The values are the means of three independent cultures. Cell densities were checked for the presence or absence of growth by optical microscopy using a Thoma counting chamber

while the archaeon *Halobacterium* sp strain NRC1 appears to be more radiosensitive in stationary phase (Kottemann et al. 2005). Consequently, the relationship between radioresistance and cellular growth phase is not conserved among archaea or radioresistant prokaryotes belonging to distinct domains of life.

Since *T. gammatolerans* was reported to withstand high doses of $\gamma$-ray, we presented the first analysis of DNA recovery kinetics after exposure to doses as high as 7,500 Gy. Prior data reported for chromosomal recovery times in *Pyrococcus* species at 2,500 Gy and in a rich-medium (DiRuggiero et al. 1997; Gerard et al. 2001; Jolivet et al. 2003b) are very close to those observed here, suggesting that no specific *T. gammatolerans* DNA repair system operates under non-saturating damage conditions. The repair mechanisms act with precision, at least at the structural level, since the reappearance of the digestion patterns was identical to that observed in non-irradiated cells. At a dose of 5,000 Gy (100% survival), the shattered chromosomal fragments ranging between 50 and 100 Kb in length are repaired in a few hours. Since archea as well as bacteria may contain several copies of chromosomes per cell, the impact of DNA repair on radioresistance is far from being negligible. However, other factors may also contribute to the radioresistance of *T. gammatolerans*. Under rich-medium growth conditions, the presence of several chromosomal fragments after irradiation at a dose of 2,500 Gy, revealed that the genome was not completely degraded. A comparable pattern was reported for several *Pyrococcus* species after an exposure to the same dose (Gerard et al. 2001) but the existence of an archaeal DNA protection system was previously refuted by comparative

studies of DNA migration patterns of several species after $\gamma$-irradiation. Nevertheless, the degree of chromosome fragmentation slightly diverged when cells were incubated in VSM-S° or ASW-S° media suggesting that compounds found in a rich-medium could help in the preservation of DNA integrity against radiation damage. For example, the viability of *P. abyssi* is improved when cells are irradiated in an organic medium as compared to a mineral medium (Gerard et al. 2001). The authors proposed a protective effect of the cystine, which contains sulphydryl residues. Additional factors can protect other cell components. Membrane pigments and KCl offer protection against cell damage in *Halobacterium* species after irradiation (Kottemann et al. 2005; Shahmohammadi et al. 1998). Accumulation of Mn(II) in radioresistant bacteria protects proteins from oxidation ensuring greater efficiency to repair the damage than in sensitive bacteria (Daly et al. 2004, 2007). Recently, it has been shown that *P. furiosus* constitutively express several detoxification systems that protect this archaeon from irradiation (Williams et al. 2007) and a ferritin-like protein induced after irradiation may bind $Fe^{2+}$ ions, thus limiting the amplification of ROS species whose are responsible of more than 80% of cell damage (Riley 1994). Therefore, ASW-S° or late VSM-S° *T. gammatolerans* cell envelope may offer a lower protection to DNA damage or cell content might be more oxidized and cells can appear more sensitive to increasing $\gamma$-ray radiation doses. However, cells pregrown in ASW-S°, can be rescued after irradiation if they are incubated in a rich-medium. These data directly confirm that cells were not killed during irradiation as in the case of *P. abyssi* (Gerard et al. 2001) and the effect of nutrient availability or energy after irradiation is also important for the survival of *T. gammatolerans* cells.

At a dose of 7,500 Gy, only 0.5% of the cells grown in VSM-S°, remained viable after irradiation. At this high dose of irradiation, a subpopulation of cells is able to repair the chromosomes but for a large majority of them, other cell damage remained and became rapidly lethal for *T. gammatolerans*.

Others factors may explain why stationary phase cells are always able to more rapidly repair the chromosomes than actively growing cells even if cells grew in the same culture medium. The variation observed is not due to a difference of growth recovery (Table 1). The chromatin composition is probably adjusted in stationary phase as described in *Thermococcus zilligii* (Dinger et al. 2000) and may change the nucleoid structure. Consequently, the chromosomes may be more tightly packed in stationary phase, limiting extensive damage as was proposed to occur in yeast (Bala and Jain 1996). It rather also may be related to a difference of chromosomal number per cell (Bernander 2003; Breuert et al. 2006; Majernik et al. 2005). A low chromosome number per cell implies less DNA lesions to be eliminated and therefore minimizes recovery times.

Our results also revealed that the evolution of the restriction pattern during the period required to repair damaged chromosomes differed between growth phases. After an incubation of several hours at 85°C, the intermediate chromosomal pattern decreased in intensity, and sizes were smaller for exponentially growing cells than for stationary phase cells. This DNA degradation was already observed for exponentially growing cells in *P. abyssi* (Jolivet et al. 2003b) as well as in *D. radiodurans* (Battista 1997; Bentchikou et al. 2007; Zahradka et al. 2006), and appears to be part of the DNA repair process. Therefore, we cannot exclude the fact that cellular repair strategies are growth phase-specific.

Under optimal growth conditions, the *T. gammatolerans* damaged DNA recovery rates appear to be slower than those reported for *D. radiodurans* (Battista 1997; Bentchikou et al. 2007; Zahradka et al. 2006). This discrepancy is more obvious when taking into account their respective sizes and genome organizations; a single chromosome of 2 Mb in *T. gammatolerans* and 4 replicons in *D. radiodurans* representing, in total, 3.28 Mb (White et al. 1999). The difference may not be ascribed to their respective generation times, since these are comparable (*T. gammatolerans*, 90 min; *D. radiodurans*, 80 min). On the other hand, the difference could be attributed to dissimilar molecular repair mechanisms in archaea, as they function with eukaryotic-like proteins.

*T. gammatolerans* cells were not always able to go into division as soon as two chromosomes per cell were repaired, since a lag took place in *T. gammatolerans* at a dose of 5,000 Gy. Exponentially growing cells exhibit asynchronous cellular division and at least 40% of the total population was in division at that stage. The impact of irradiation on this asynchronous population is not the same than that for stationary cells in which replication and division events are sporadic, especially when cells are irradiated at a high dose. Consequently, the recovery of exponentially growing cells restart according to their position in the cell cycle interrupted by irradiation and DNA repair is not the last event of the recovery from gamma irradiation at high doses.

Interestingly, DNA repair rate seems not to be the most important factor in *T. gammatolerans* radioresistance, as supported by our experiments performed under conditions leading to retarded DNA repair kinetics while retaining identical surval rates. Cells do not seem to stick to tube walls, since the cell density is the same during growth recovery indicating that they remain in suspension. During repair, *P. abyssi* cells export damaged components into the medium (Jolivet et al. 2003b). In our experimental conditions, these toxic components would then be less diluted in Hungate tubes than in serum bottles, thus increasing the time required to repair all cell damage. Then, rapid DNA

repair is not a prerequisite to ensure radioresistance of *T. gammatolerans*.

Comparative studies between radioresistant organisms and between resistant and sensitive archaea to ionizing radiation will be useful to gain insights into the molecular basis of radioresistance.

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