- Supplementar

y Information -

1. Hypotheses and the experimental strategy

A priori, there are six known mechanisms that, alone or in some combination, could rejoin hundreds of partially overlapping chromosomal fragments in *D. radiodurans*:

(i) Non-homologous end-joining (NHEJ) of DNA fragments[30,31]

(ii) Homologous recombination (HR) via conservative crossovers involving ends of overlapping fragments[16,32,33]

(iii) Single strand annealing (SSA) via a strand-biased 5'-exonucleolytic erosion of the two ends resulting from a DSB providing a 3'single strand overhang for eventual annealing with (a) each other (intra-chromosomal SSA) via an internal micro-homology (usually a tetranucleotide) resulting necessarily in the deletion of the sequence between two micro-homologies[33], or (b) a fully complementary single strand end of a contiguous fragment belonging to another chromosomal copy (inter-chromosomal SSA)[16] thereby avoiding sequence deletions (SSA in Fig. 4)

(iv) Synthesis dependent strand annealing (SDSA) can assemble DNA fragments by strand invasion, i.e., annealing of both recessed single-stranded ends to the same intact region of a homologous chromosome (D-loop formation; the bracketed intermediate in Fig. 4). D-loop provides the primer for synthetic elongation of the annealed 3' end to produce a moving D-loop, like in transcription. Once elongated on the same template sequence, the two extended complementary ends dissociate from their template and anneal[33,34].

(v) Break-induced replication (BIR) is, in its early stage, mechanistically akin to an

SDSA event involving only one broken end[33,35]. Following the initial one-end strand invasion (D-loop formation), either (a) a genuine replication fork is formed resulting in a semi-conservative copying of the template, or (b) the displaced newly synthesized single strand (like in SDSA, Fig. 4), is itself copied by a discontinuous synthesis resulting in a conservative replication of the homologous DNA template. Either version of BIR could link together many DNA fragments by iteration of the replication process at the growing ends, which would leave little, if any, original double-stranded material in the repaired chromosome.

(vi) Copy choice (CC) is a DNA replication mechanism that involves sequential template switching between double-stranded templates from different overlapping fragments until a full-size chromosome is newly synthesized[36]. This mechanism is not clearly distinguishable from iterative BIR. In its original version, it is unidirectional and does not require DNA breakage for template switching.

Mechanisms involving extensive homologies, (ii), (iii b), (iv), (v) and (vi), can assure a high precision of chromosomal reconstruction, others can be (i), or must be (iii a), mutagenic. Mechanisms (i) and (ii) involve no significant DNA synthesis, whereas (iii a and b) and (iv) involve significant but limited synthesis, (v) requires extensive, close to total, synthesis, and in (vi) all repaired DNA is newly synthesized.

2. Supplementary Results

Density gradient analysis of the structure of repaired chromosomes

The repair of the *D. radiodurans* DNA shattered by ionizing radiation was analysed by an adaptation of the classical Meselson-Stahl experiment[17], i.e., by DNA density labelling and analysis of its buoyant density by ultracentrifugation in CsCl equilibrium density gradients. The extent and pattern of DNA repair synthesis was monitored by the incorporation of a non-

radioactive heavy analogue of thymidine (5-bromo-deoxyuridine – 5-BrdU) present only after irradiation, for 3 h, when the bulk of DNA repair appears to be completed (Fig. 1a). The incorporation of 5-BrdU does not hinder DNA repair (see Fig. 2). The radioactive labelling of DNA by ^{3}H-thymidine incorporation was carried out under three different regimens: (a) "pre-labelling", continuous labelling only before irradiation, (b) "post-labelling", labelling only after irradiation during the 3 h period of DNA repair, and (c) "pre- and post-labelling", i.e., combined (a) and (b).

The DNA density analysis from unirradiated *D. radiodurans* cultures (Supplementary Fig. S1c) agrees with the Meselson-Stahl experiment with *E. coli*, i.e., the replication of all genomic components appears to be semi-conservative: the H/L (heavy/light) density appears after the first replication cycle, H/H during the second. As expected, upon DNA denaturation and centrifugation in alkaline CsCl gradients, all single-stranded material segregates into either H or L densities (Fig. S1, a1-c1). Because DNA is monitored by ^{3}H-thymidine radioactivity, the unirradiated cultures show, in the "pre-labelling" regimen, only the H/L* material (* denotes ^{3}H-label) (Fig. S1a), and only L* in denaturing gradients (Fig. S1a1). In the "post-labelling" regimen predominantly H*/L and – to the extent of second replication round – also H*/H* are found (Fig. S1b) (and only *H in denaturing gradients, Fig. S1b1). We shall call the light DNA synthesized before irradiation "old" and the heavy DNA synthesized after irradiation "new". Because only old DNA is damaged in radiation experiments and the repair takes place in the heavy medium, the two strands of the DNA repaired by NHEJ and HR are expected to be essentially old/old, those repaired by BIR substantially old/new and/or new/new, and those made by CC fully old/new and/or new/new (see above). None was observed. In neutral density gradients, the old "pre-labelled" DNA was found spread towards intermediate densities due to 5-BrdU incorporation after irradiation (Fig. S1a). On the other hand, the "post-labelled" new DNA synthesized after irradiation is

essentially heavy with significant skewing towards lighter densities (Fig. S1b). Thus, the density patterns of DNA repaired after gamma radiation do not resemble either semi-conservative or conservative replication and therefore do not support NHEJ, HR, CC or BIR as the predominant DNA repair mechanism (text above and Fig. S1, a-c). Yet the amount of DNA repair synthesis (Fig. 1) and the extent of density shifts (Fig. S1, a-c) do not support standard SSA or SDSA mechanisms either, but rather some kind of DNA repair creating large patchworks of old and new DNA material. To test for an old/new patchwork structure at the level of single strands, the density of denatured repaired DNA was analysed in alkaline CsCl density gradients. The densities of pre-labelled single strands are shifted substantially from light (old) towards heavy (new) (Fig. S1c1). Because the size of the DNA isolated by the described method is in the range of 15 to 25 kb (not shown), i.e., generally smaller than the in vivo size of fragments following 7 kGy irradiation (20-30 kb), the detection of fragments containing old and new material is diminished.

What is the size of newly synthesized DNA patches? Fig. S1a1 shows that, in the "pre-labelling" regimen, the majority of single stranded material is heavier than light (old) but lighter than heavy (new). The inverse is not true. In the "post-labelling" regimen, the single-stranded DNA is under the heavy peak and skewed towards lighter densities (Fig. S1b1). In other words, during repair in the presence of 5-BrdU, the old light strands are made heavier (by association with new heavy material) than are the new heavy strands made lighter (by association with old light material). This suggests that the tract of newly synthesized (density and radioactively labelled) material is often longer than the size of original light (radioactively but not density labelled) material. This DNA density analysis suggests a "distributive" mode[17] of DNA (repair) replication that generates a patchwork structure of old light radiation-produced fragments interconnected by newly synthesized heavy DNA blocks that are at least as long as the initial fragments. The old and new DNA blocks are connected

via old/new hybrid DNA regions detected as H/L fragments. These results exclude all repair mechanisms involving no, or very limited, DNA synthesis, as well as those requiring complete, or close to complete, DNA synthesis.

3. Supplementary Discussion

Analysis of the results and general discussion

D. radiodurans is a small non-sporulating and non-pathogenic bacterium whose sequenced genome is composed of two circular chromosomes (2.65 and 0.41 mega bp) and two circular plasmids (0.177 and 0.046 mega bp)[9]. Its extreme radiation resistance appears as a by-product of natural selection for its desiccation resistance[4]. Each of the 41 tested *D. radiodurans* radiation sensitive mutants are also desiccation sensitive proportionally to their radiation sensitivity[4] and selection for desiccation resistance in other bacteria co-selects radiation resistance[5]. This correlation implies that, although excessive desiccation and ionizing radiation cause damage to all key cellular components, genome reconstitution rescues cellular life.

We interpret the results of our experiments as evidence that the capacity of *D. radiodurans* to repair hundreds or thousands of double strand breaks in its genome is due to a mechanism that Matthew Meselson suggested we call extended synthesis dependent strand annealing, ESDSA. ESDSA differs from the standard "limited" SDSA in that it involves strand invasion (the bracketed intermediate in Fig. 4) between dispersed fragments belonging to different chromosomal copies and sharing overlapping sequence homology. The synthetic extension of the priming 3' termini proceed probably to the end of the invaded fragments, followed by the annealing of complementary newly synthesized strand extensions of contiguous fragments from other chromosomal copies (Fig. 4).

The distinct features of the ESDSA model are that (i) it requires at least two

chromosomal copies broken at different positions, and (ii) it involves a single-round multiplex PCR-like step (steps 2 and 3 in Fig. 4) resulting in long, newly synthesized single strand overhangs that allow for an accurate annealing process. For consistency, the standard SSA mechanism (Fig. 4) should be called synthesis-independent strand annealing, or SISA, because no synthesis is required for the annealing step, only for the eventual gap filling after annealing (Fig. 4). Such mechanism was proposed by Daly and Minton[16]. A SISA mechanism with an extensive nick translation synthesis is a conceivable alternative to the ESDSA. However, the *E. coli* PolA Klenow fragment complements *D. radiodurans polA* mutants for resistance to γ-radiation[37], showing no requirement of the PolA nick translation activity for DNA repair. Furthermore, this alternative "cycling" SISA mechanism would produce almost completely newly synthesized DNA that is incompatible with our results (Figs. 2b and S1). Similarly, iterative BIR would also produce only semi-conservative (v, a) or conservative (v, b) DNA replication leaving little, if any, original DNA duplex within the repaired chromosome. A combination of the (v, b) version of BIR and HR is conceivable, yet would not explain the massive growth of DNA in the *recA* mutant (Fig. 1e). The apparent regularity of the alternation of old and new DNA blocks within the repaired chromosomes (Fig. 2) can be readily explained by the ESDSA model. Although many details of the ESDSA mechanism have not been clarified, e.g., the priming step in DNA strand elongation, the major simple alternatives have been ruled out.

Both DNA synthesis and repair are less efficient in the *recA* mutant (Fig. 1e, f) than in the wild type (Fig. 1a, b). Therefore, it is conceivable that deinococcal RecA, through its preferential double-strand binding activity[12], brings together the recessed DNA fragments with overlapping homology, thereby facilitating an accurate priming of strand extension in ESDSA (Fig. 4a and the text above). However, the key involvement of RecA is clearly in the chromosomal maturation process (Fig. 4b).

A *polA*, but not *recA,* mutant is fully deficient in ESDSA repair (Fig. 1). However, DNA base and sugar damages by radiation-induced oxygen free radicals, which can cause single-strand breaks directly and by proximal base excision repair (BER) events, are at least 10 times more frequent than DSBs[21] and typically require PolA for the completion of repair[22]. Therefore, it is expected that PolA protects the integrity of the DNA fragments produced by γ-radiation (see the disappearance of DNA fragments in the *polA* mutant, Fig. 1c) by repairing single nucleotide gaps created by the BER enzymes, and that it participates directly in strand extension synthesis (Fig. 4a). However, before the isolation of a conditional mutant of DNA polymerase III, we cannot exclude the possibility that PolA only initiates a DNA polymerase III-catalysed single strand elongation or even contributes only to the maintenance of fragments.

Unlike other bacteria, all tested radiation resistant bacterial species show condensed nucleoids even after ionizing radiation[15]. It was suggested that a special ring-like chromatin holds broken DNA ends in register and facilitates correct repair by NHEJ[14]. However, a high concentration of DNA fragments in the condensed nucleoid is expected to facilitate any bimolecular homologous interaction required for DNA repair, including ESDSA. Perhaps, in addition to the peculiarity of the RecA[12], the condensed chromatin is another evolutionary innovation of *D. radiodurans* assuring its radiation resistance through a high efficiency ESDSA repair.

Can ESDSA account for the apparent fidelity of DNA contig assembly in *D. radiodurans*? The problem of incorrect fragment assembly via repetitive sequences present in single strand tails could be circumvented in ESDSA if the synthesis of complementary single strands were coincident in space and time, as discussed above. But even for solitary strand extensions (Fig. 4a), the accuracy can be maintained if the fragments' tails were much longer than the longest repetitive sequences. In that case, annealing only a limited repeated sequence

block within a long non-complementary single-stranded overhang could not readily link the two fragments.. The avoidance of a lethal assembly of non-contiguous DNA fragments may have provided sufficient selective pressure for the evolution of the ESDSA mechanism (with long single stranded overhangs) rather than a mechanistically more simple inter-chromosomal SISA (with shorter overhangs) (Fig. 4a).

The high fidelity ESDSA process requires that all fragment's overhangs be extended by copying a fragment that is contiguous in the intact chromosome. Each mispriming of strand elongation, e.g., within a repeated sequence of a wrong fragment, will later cause the annealing of two non-contiguous fragments generating a gross chromosomal rearrangement. We can think of four strategies for assuring the fidelity of both priming of the single strand synthesis and of strand annealing: (1) homologous pairing of recessed double-stranded DNA fragments before the initiation of D-loops, perhaps by the peculiar deinococcal RecA[12], (2) editing the pairing process by mismatch repair proteins[24,25], (3) repeat-binding proteins[9] preventing sequence repeats from becoming single-stranded or from annealing, and (4) stable secondary structures (hairpins) of repetitive sequences[23] preventing their annealing with another partner molecule. Strategy (1) predicts that DNA assembled by fragment concatenation in the dead *recA* mutant cells (Fig. 1e) could be riddled with rearrangements.

Some ramifications of this work. The extraordinary diversity of genetic lineages represented in the *D. radiodurans* genome (*Bacillus*, *Thermus* and multiple eukaryote-like genes)[9,23] might be a hallmark of an efficient incorporation of foreign genes in its evolutionary past, probably due to the combination of its high transformability and recombination repair.

If we consider growing *E. coli* as a bacterial paradigm of renewing epithelia (including the evolution of carcinomas), then *D. radiodurans* can be a bacterial paradigm of

long-lived, non-dividing neurons. Therefore, exploring mechanisms of deinococcal robustness could inspire new approaches in regenerative medicine. Moreover, evolving highly robust bacteria could provide an option for spreading life on other planets by directed panspermia[38].

4. Supplementary Methods

Bacterial strains, growth conditions, and gamma irradiation. The following *D. radiodurans* strains were used: R1 (ATCC 13939) wild type[39], GY10922 Δ(*cinA-recA*)$_1$::kan[40], and IRS501 *polA* (J. R. Battista). A thymine-requiring (*thy*$^-$) derivative of the R1 strain was isolated by selection on a solid minimal medium containing thymine (50 μg/ml) and trimethoprim (100 μg/ml)[41].

Bacteria were grown in TGY broth (0.5 % tryptone, 0.1 % glucose, 0.15 % yeast extract) at 30^{o}C to the late exponential phase (OD_{650}=0.6 - 0.8). Cultures were washed in 10 mM sodium phosphate buffer, concentrated 10 times in the same buffer, and irradiated on ice with a ^{60}Co γ ray source at a dose rate of 11 Gy/s. A dose of 7 kGy was applied to the cells in all irradiation experiments resulting in 80 to 90% survival of the wild type strain. The number of viable cells was estimated by plating serial dilutions onto TGY plates and the colonies were counted after 3-4 days incubation at 30^{o}C.

Kinetics of DNA repair measured by pulsed-field gel electrophoresis. Irradiated cultures were diluted in TGY to an OD_{650}=0.2 and incubated at 30^{o}C. At indicated intervals, 5-ml samples were taken to prepare DNA plugs as described by Mattimore and Battista[4]. The DNA contained in the plugs was digested with 60 units of NotI restriction enzyme (Roche) for 16 h at 37^{o}C. After digestion, the plugs were subjected to pulsed-field gel electrophoresis in 0.5xTBE using a CHEF-DR III electrophoresis system (Bio-Rad) at 6 V/cm^2 for 20 h at 14°C, with a linear pulse ramp of 50-90 s and a switching angle of 120^o.

Rate of DNA synthesis measured by DNA pulse labelling. Unirradiated and irradiated exponentially growing cultures were incubated and 0.5-ml samples taken and mixed with a 0.1 ml pre-warmed TGY medium containing 6 μCi ^{3}H-thymidine (Amersham; specific activity 86 Ci/mmol). Radioactive pulses were terminated after 15 min by addition of 2 ml ice-cold 10% TCA. Samples were kept on ice for at least 1 h, and then collected by suction onto Whatman GF/C filters followed by washing with 5% TCA and 96% ethanol. Filters were dried overnight at room temperature, and placed in 5 ml scintillation liquid. The precipitated counts were measured in a liquid scintillation counter (Wallac, Pharmacia).

Radioactive and density DNA labelling. *D. radiodurans thy*$^{-}$ cells were radioactively and density labelled during growth in the presence of ^{3}H-thymidine and 5-bromo-2'-deoxyuridine (5-BrdU), respectively. Density labelling was performed in all experiments by adding 5-BrdU to the medium only after irradiation. The radioactive labelling was performed in three different regimens. (a) "Pre-labelling": cells were grown overnight in TGY supplemented with 5 μCi/ml ^{3}H-thymidine. They were collected by centrifugation, washed twice in the phosphate buffer, concentrated 10 times in the same buffer, and exposed to 7 kGy γ radiation. Both irradiated and unirradiated cultures were diluted to an OD_{650}=0.2 in TGY containing 20 μg/ml 5-BrdU. The unirradiated culture was grown in 5-BrdU-supplemented TGY for 2.5 h (corresponding to one mass-doubling), whereas the irradiated culture was grown for 3 h (the time required for DSB repair to be completed) at 30^{o}C. (b) "Post-labelling": cells were grown overnight in non-radioactive TGY, and radioactivity (20 μCi/ml ^{3}H-thymidine) was added to the 5-BrdU-supplemented TGY only after irradiation. The experimental procedure was otherwise the same as described for "pre-labelling". (c) "Pre- and post-labelling": the procedure was a combination of (a) and (b), i.e. the cells were radioactively labelled before and after irradiation.

DNA preparation and density gradient analysis. Radioactively and density labelled DNA

was isolated from *D. radiodurans* by the use of the Qiagen DNeasy Tissue Kit according to the supplier's instructions. The DNA was centrifuged to equilibrium in a neutral or alkaline cesium chloride (CsCl) solution (1.7246 g/ml) in a VTi90 rotor (Beckman) for 24 h at 40.000 rpm and 20^{o}C. To obtain the desired CsCl concentration, the refractive index of CsCl solution was adjusted (by adding water) to 1.4030 for neutral gradients, and to 1.4050 for alkaline gradients. For alkaline gradients, the DNA was denatured by 10 min heating in boiling water, followed by chilling in ice water, and the CsCl solution was adjusted to pH 11.8. Gradients were collected from the bottom of pierced tubes (OptiSeal 4.9 ml, Beckman) in about 25 (12-drop) fractions. 100-µl aliquots of fractions were applied on round filters, dried under the lamp for several hours, and the radioactivity was measured in a scintillation counter.

UV-induced photolysis of BU-substituted DNA. *D. radiodurans thy*$^{-}$ culture was grown and irradiated with 7 kGy γ radiation as described above. The irradiated culture was diluted to an OD_{650}=0.2 and grown in 5-BrdU-supplemented TGY for 3 h. The cells were collected by centrifugation, resuspended in the phosphate buffer and incubated (starved) in the buffer for one hour at 30^{o}C. Cell suspension was cooled in ice and exposed in a thin layer to indicated doses of 254-nm UV light. Both UV-irradiated and unirradiated cells were embedded in agarose plugs for DNA analysis by PFGE (see above).

Immunofluorescent microscopy of cellular DNA synthesis. Exponentially grown *D. radiodurans thy*$^{-}$ culture was harvested by centrifugation (6000 g), concentrated 10 times in a phosphate buffer (66.7 mM, pH 6.8), and irradiated with 7 kGy γ rays. Irradiated and unirradiated cells were diluted to OD_{650}=0.2 in TGY and incubated at 30^{o}C. At different post-irradiation time points cells were pulse-labelled with 5-BrdU (20 µg/ml; Sigma) for 10 min and fixed with 75% methanol at 4^{o}C overnight. Fixed cells were washed with PBSTE (PBS with 10 mM Tris-HCl and 1 mM EDTA; wash buffer) and treated with lysozyme (2 mg/ml;

Sigma) for 10 min. Cells were then washed twice and blocked with 2% w/v bovine serum albumine (Sigma) in PBSTE (PBSTE-BSA) for 15 min. Half of the cells were treated with 4 M HCl for 1 h at 37 ^{o}C and washed twice (denatured samples). Detection of 5-BrdU was subsequently performed via indirect immunofluorescence. Cells were incubated in anti-mouse anti-BrdU monoclonal antibody (Becton Dickinson) diluted in PBSTE-BSA (4 μg/ml) for 1 h at room temperature with gentle shaking, washed twice and incubated in goat anti-mouse IgG-FITC (Sigma) diluted in PBSTE-BSA (12 μg/ml) for 1 h at room temperature with gentle shaking. Cells were then washed twice and mounted onto an agarose film on a glass slide. Native and denatured cell samples were examined with the Zeiss Axioplan 2 fluorescence microscope. Phase contrast and fluorescence images were taken at 100 x magnification. Image analysis was performed using Metamorph software by measuring average foci fluorescence per cell for 10 000 to 35 000 cells for each condition.

Supplementary Notes (References)

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