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Accuracy of Genome Reassembly in γ-Irradiated *Escherichia coli*

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Summary

γ-Radiation, a powerful DNA-damaging agent, can often lead to the formation of genome rearrangements. In this study, we have assessed the capacity of *Escherichia coli* to accurately reassemble its genome after multiple double-strand DNA breaks caused by γ-radiation. It has recently been shown that very high doses of γ-radiation or RecA protein deficiency cause erroneous chromosomal assemblies in *Deinococcus radiodurans*, a highly radiation-resistant bacterium. Accordingly, we have examined the accuracy of genome reassembly in both wild-type and *recA* strains of *E. coli* after exposure to the doses of γ-radiation which reduce the survival by 10^6- to 10^7-fold. Thirty-eight percent of wild-type survivors showed gross genome changes, most of which were found to be the consequence of the excision of e14, a 15-kb defective prophage. Only one additional type of gross genome rearrangement was detected, presumably representing the duplication of a DNA fragment. These results demonstrate an unexpectedly accurate genome reassembly in wild-type *E. coli*. We have detected no genome rearrangements in *recA recBCD* and *recA recBCD sbcB* mutants, suggesting that RecA-independent DNA repair in *E. coli* may also be accurate.

Key words: Escherichia coli, DNA repair, double-strand DNA breaks, genome rearrangements, γ-radiation, *recA* mutants

Introduction

γ-Radiation, a type of ionizing radiation, damages various cell parts, directly or through the induction of oxidative stress. Protein oxidation and double-strand breaks (DSBs) in the DNA are among its most detrimental effects. As a germicidal agent, γ-radiation is widely used in food technology to eliminate microbiological contamination, thus improving food safety as well as food shelf life (*1*,*2*). In addition, it has a capacity to alter epitopes of the food allergens, which can be applied to reduce allergenicity of foods (*3*). γ-Radiation is also a potent mutagen, and is therefore used in biotechnology for generation of new microbial strains characterized by increased production of commercially relevant compounds (*4*).

Some bacteria have developed highly effective mechanisms of defense against ionizing radiation. For example, extreme radiation resistance of *Deinococcus radiodurans* can be ascribed to its ability to protect proteins from oxidation (*5*,*6*) and to an accurate and efficient repair of its genome shattered by hundreds of DSBs (*7*,*8*). *Escherichia coli* does not possess such efficient protection against oxidative damage. Also, the primary DSB repair mechanism differs considerably between *E. coli* and *D. radiodurans* (*7–11*). In wild-type *E. coli*, DSBs are repaired by homologous recombination (HR) that is initiated by the RecBCD enzyme (a set of reactions known as the Rec BCD pathway) (*11–14*). In contrast, *D. radiodurans* is naturally devoid of RecB and RecC proteins (*15*), and consequently, of the RecBCD recombinational pathway. The first stage of DSB repair in this radiation-resistant bacterium proceeds by a special kind of extended synthe-

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sis-dependent strand annealing (ESDSA) (*7*,*8*) in which RecFOR pathway enzymes play a dominant role (*10*). Hence, *E. coli* and *D. radiodurans* are expected to repair multiple DSBs caused by γ-radiation with a different level of success.

We have recently identified conditions that impair remarkable fidelity of DSB repair during genome reassembly in *D. radiodurans* (*16*). High doses of γ-radiation that cause a strong reduction in survival seem to be a prerequisite for the formation of gross DNA rearrangements in wild-type *D. radiodurans*, as detected by pulsed--field gel electrophoresis (PFGE). Moreover, deletion of *recA*, a gene for the main bacterial recombinase, makes the *D. radiodurans* genome more prone to both spontaneous and γ-radiation-induced rearrangements (*16*). The RecA protein plays a central role in homologous recombination and recombinational DNA repair in bacteria (*11*,*17*,*18*). It binds onto single-stranded DNA substrate and catalyzes the key recombination reaction – homologous pairing and DNA strand exchange. RecA is highly conserved among bacteria and has archaeal and eukaryotic homologues (*19*). Interestingly, the *D. radiodurans* RecA protein shows mechanistic differences when compared to the *E. coli* RecA; *in vitro* it preferably binds to double-stranded DNA and promotes an inverse DNA strand exchange reaction (*20*). RecA is clearly crucial for the efficient reconstitution of an intact *D. radiodurans* genome after γ-irradiation (*7*,*8*). In the first stage of the reconstitution process, called ESDSA, both ends of chromosomal fragments produced by radiation acquire long single-stranded tails by strand-elongation synthesis using overlapping homologous chromosomal fragments as templates (*7*). Newly synthesized single-strand extensions subsequently anneal with high precision, joining together contiguous DNA fragments into long double--stranded linear intermediates. In the second stage, the long intermediates formed by ESDSA are assembled into circular chromosomes by RecA-dependent homologous recombination (*7*). In addition to its role in the final stage of the repair process, recent study has suggested a role for the deinococcal RecA protein also in ESDSA, *i.e.* in the resection of DNA ends and in the efficient priming of the repair synthesis (*8*).

The presence of repetitive stretches of DNA in the genome represents an obstacle to accurate genome reassembly by HR after multiple DSBs (*21*,*22*). For example, in *Saccharomyces cerevisiae*, chromosomal rearrangements after γ-irradiation have been attributed to recombination between non-allelic repetitive DNA sequences (*23*). Also, *D. radiodurans* seems to have hotspots resulting in distinctive types of gross chromosomal rearrangements (*16*). One type of DNA rearrangements in *D. radiodurans* – integration of the small plasmid into the large chromosome – was found to be caused by two copies of an insertion sequence present on two chromosomal elements (*16*). The number of insertion sequences and small noncoding repeats in *D. radiodurans* and *E. coli* is similar (*15*,*24*), and so is the rate of DSB formation after γ-irradiation (*25*,*26*).

In this work, we have examined the fidelity of DSB repair during genome reassembly in γ-irradiated *E. coli*. Also, since the RecA protein was shown to play a key role in avoiding gross chromosomal rearrangements during DSB repair in *D. radiodurans* (*16*), we have tested here the effect of the *recA* mutation on the fidelity of the repair process in *E. coli*. Interestingly, the *D. radiodurans* genome does not code for RecBCD and SbcB (ExoI) nucleases (*15*). Moreover, it has been shown that expression of *E. coli sbcB* and *recBC* genes in *D. radiodurans* reduces its radiation resistance (*27*,*28*). To make the repertoire of DNA repair functions of *E. coli* more similar to that of *D. radiodurans*, we have constructed *recA recBCD* and *recA recBCD sbcB* mutants of *E. coli* and tested them for genome rearrangements following γ-irradiation. The inactivation of RecBCD was necessary also to prevent 'reckless' DNA degradation that occurs in *E. coli recA* mutants exposed to DNA-damaging agents, and which is carried out by the nucleolytic (ExoV) activity of RecBCD (*29*–*31*). 'Reckless' DNA degradation may lead to the complete loss of the cellular DNA (*30*,*31*), thus making DNA analysis in affected cells practically impossible.

We have analyzed DNA restriction patterns of *E. coli* wild-type and *recA recBCD* (*sbcB*) cells after exposure to γ-radiation reducing survival by 10^6- to 10^7-fold (the same low level of survival was used in previous experiments with *D. radiodurans* (*16*)). Thirty-eight percent of wild-type survivors showed significant changes in their DNA NotI restriction patterns, most of which correspond to the excision of the 15-kb prophage e14. No genome rearrangements were detected in *recA recBCD* and *recA recBCD sbcB* mutants, demonstrating the ability of *E. coli* RecA-independent DNA repair system to provide correct genome reassembly.

Materials and Methods

Bacterial strains, media and growth conditions

The *E. coli* strains used in this work are listed in Table 1 (*31*–*34*). Bacteria were grown at 37 °C in Luria--Bertani (LB) liquid medium with aeration or on LB plates (*35*). P1 transduction method (*35*) and selective LB plates supplemented with tetracycline (10 μg/mL) or chloramphenicol (15 μg/mL) were used for the construction and isolation of new strains. The *recA* transductants were checked for their UV sensitivity phenotypes.

Table 1. *Escherichia coli* strains used in this work

Strain[a]	Relevant genotype	Source or reference
MG1655	wild type	(*32*)
LMM1718	Δ(*recC-argA*)*234*[b]	(*33*)
JJC889	Δ*sbcB*::*cam*	(*34*)
LMM1245	*recA*269::Tn10	(*31*)
LMM1863	Δ(*recC-argA*)*234*[b] Δ*sbcB*::cam	P1.JJC889 × LMM1718 to Cm^r
LMM2368	Δ(*recC-argA*)*234*[b] *recA*269::Tn10	P1.LMM1245 × LMM1718 to Tc^r
LMM2369	Δ(*recC-argA*)*234*[b] Δ*sbcB*::cam *recA*269::Tn10	P1.LMM1245 × LMM1863 to Tc^r

[a]Strains are derivatives of MG1655, except JJC889 and LMM1245, which are derivatives of AB1157 and were used for the construction of other strains
[b]The deletion encompasses *recC*, *ptrA*, *recB*, *recD* and *argA* genes

γ -Irradiation

Bacteria were grown overnight, diluted 500-fold in fresh LB medium and grown to an $A_{600\ nm}$ of 0.2 to 0.4. Prior to irradiation, bacteria were washed with 10 mM sodium phosphate buffer and resuspended in 1/10 volume of the same buffer. Irradiation was performed on ice with a ^{60}Co γ-ray source at a dose rate of 4.5 Gy/s. Appropriate dilutions of the cells in phosphate buffer were plated on LB plates to obtain individual colonies. Viability of irradiated samples was expressed relative to the viability of unirradiated aliquots of the same samples. On the basis of survival curves obtained (Fig. 1), the following doses reducing survival 10^6- to 10^7-fold were chosen for further experiments: 3.2 kGy for the wild type, 360 Gy for the *recA recBCD* strain, and 480 Gy for the *recA recBCD sbcB* strain. After 2–3 days of incubation at 37 °C, the colonies of wild-type and mutant survivors were picked for further DNA analyses. Since the colonies of survivors varied in size to a certain extent, the colonies of different sizes were picked in order to obtain a representative sample of surviving clones. The γ-resistance phenotype of wild-type survivors was additionally tested, by the above protocol, at the dose of 2 kGy. Cultures of survivors as well as the unirradiated culture used as a control were grown from a single colony.

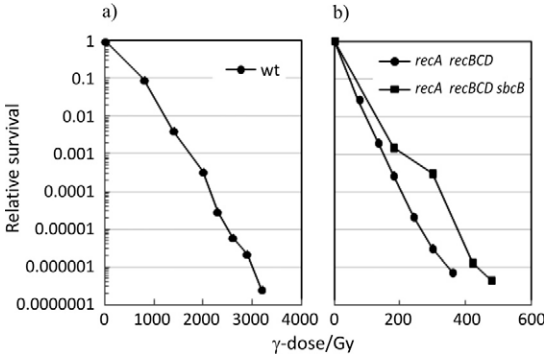

Fig. 1. Representative survival curves for: a) *E. coli* wild-type (wt) and b) *recA recBCD* and *recA recBCD sbcB* strains after γ-irradiation. The number of colony-forming units per mL of each irradiated culture is expressed relative to the number of colony-forming units per mL of the respective unirradiated (control) culture

DNA analysis of individual colonies by pulsed-field gel electrophoresis

Colonies obtained after irradiation were inoculated into the fresh LB medium and grown to the stationary phase ($A_{600\ nm}$ of approx. 2). Aliquots of 200 μL of cell cultures were sequestered for the preparation of pulsed-field gel electrophoresis (PFGE) samples. The cells were pelleted, washed twice with 200 μL of 0.5 M EDTA (pH= 8.0) and resuspended in 0.05 M EDTA before being embedded in an equal volume of 1.6 % low-melting-point ultra-pure agarose (Gibco, Life Technologies, Invitrogen, Carlsbad, CA, USA) in 0.05 M EDTA. Agarose plugs with embedded cells were soaked overnight at 37 °C in a solution of 0.05 M EDTA containing lysosyme (1 mg/mL), lauroyl sarcosine (0.5 %) and sodium deoxycholate (0.2 %). This solution was replaced by the 0.5 M EDTA (pH=8.0) solution containing proteinase K (1 mg/mL) and lauroyl sarcosine (1 %) and left overnight at 50 °C. The DNA-containing agarose plugs were washed twice in 0.5 M EDTA and three times in Tris-EDTA (TE) buffer, and then further treated with the restriction enzyme NotI (30 U) or SpeI (30 U) (New England Biolabs, Ipswich, MA, USA). PFGE was performed in 0.5×TBE buffer using a CHEF-DR III electrophoresis system (Bio-Rad, Hercules, CA, USA) under the following conditions: 6 V/cm^2 for 22 h at 12 °C, with a linear pulse ramp of 10–60 s and a switching angle of 120°. PFGE-resolved DNA was stained with ethidium bromide and visualized under UV illumination.

PCR confirmation of e14 prophage presence/excision

Primers inE14 left (5'-GCGGTATTCGCTCTCTGAAC-3') and inE14 right (5'-TCCTCCAGGAGAAAGCAAAA-3'), located within the e14 prophage DNA sequence, were used as an indicator of e14 presence (expected PCR product size 1792 bp). Alternatively, primers e14 left (5'-CATAACGACGCAATGTGGAC-3') and e14 right (5'-ATGGCTCGATCAAGAACACC-3'), separated by the 15-kb e14 DNA sequence, were used for the confirmation of e14 excision (expected PCR product size approx. 1100 bp). Primer positions on the chromosome are shown in Fig. 2 (*36*,*37*). PCR was performed on *E. coli* cells ad-

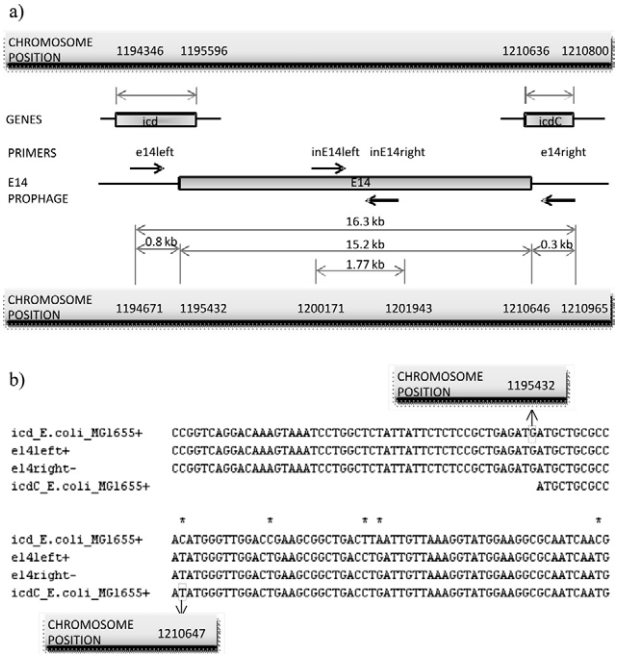

Fig. 2. Analysis of the e14 prophage excision site: a) positions of primers used in the PCR analysis presented in Fig. 5. Primers are shown as thick arrows and relevant DNA features (genes, prophage) as rectangles. Chromosome position of e14 borders was taken from Harris *et al.* (*36*); b) PCR products obtained in various e14-missing survivors by primers e14 left and e14 right were sequenced, and found not to vary in the site of e14 excision. The figure shows a segment of the alignment of these PCR products with *E. coli* genes *icd* and *icdC*, bordering the e14 prophage. Symbols + or – next to the sequence names mark the obtained DNA sequence or its reverse complement. Prophage e14 carries a sequence that is homologous to the C-terminus of the *icd* gene. After e14 insertion, this sequence replaces the native C-terminus. The native terminus instead ends up at the opposite end of the prophage as the *icdC* pseudogene (*37*). The two termini can be distinguished by point mutations marked by asterisks above the sequences in panel b

justed to the same concentrations (as guided by $A_{600\ nm}$ measurement), with HotStar DNA polymerase (Qiagen, Hilden, Germany). Conditions of 95 °C for 20 s, 57 °C for 30 s and 72 °C for 3 min were cycled 30 times.

Sequencing of the e14 prophage excision region

Primers e14left and e14right were used to amplify the region spanning the prophage e14 excision site by Phusion Hot Start II High-Fidelity DNA Polymerase (Finnzymes, Thermo Scientific, Pittsburgh, PA, USA). PCR conditions of 98 °C (denaturation), 57 °C (annealing) and 72 °C (elongation) were cycled 40 times, and the resulting PCR product was purified with the Wizard SV gel and PCR clean-up kit (Promega, Madison, WI, USA). Sequencing was performed (using the same primers) by the Macrogen Europe sequencing service (Amsterdam, the Netherlands).

Detection of repetitive sequences bordering the e14 prophage sequence

To estimate the probability of excision of the e14 prophage by the cell DNA reparatory machinery, DNA sequences bordering the e14 prophage were examined for the presence of repetitive sequences. The location of e14 ends was taken from Harris *et al.* (*36*) and used for the extraction of 10-kb DNA sequences located ±5 kb from the edges of the prophage. These sequences were scanned by the Mummer program (*38*) in search for the exact repeats that occur in both sequences.

Results

Accuracy of genome reassembly after γ-irradiation in wild-type E. coli

A representative survival curve for γ-irradiated wild-type *E. coli* is shown in Fig. 1a. In this particular experiment, we isolated colonies of survivors of 3.2 kGy for subsequent DNA analysis. This dose reduced colony-forming ability of *E. coli* from $1.3 \cdot 10^9$ to $3.2 \cdot 10^2$ colony-forming units per mL (CFU/mL). This is the same low level of survival (10^{-7} to 10^{-6}) at which we have previously detected gross rearrangements in *D. radiodurans* genome after exposure to 25 kGy (*16*), thus making the experiments with two different organisms comparable.

Thirty-seven *E. coli* colonies formed by the cells that survived 3.2 kGy of γ-radiation were regrown and examined for possible changes in their DNA NotI restriction pattern (Fig. 3). Fourteen survivors with changed NotI patterns were detected (Fig. 3, compare samples no. 1–37 with the unirradiated control designated C). Thirteen of them displayed the same change in the NotI pattern – a loss of mass in the 98.6-kb DNA fragment, indicating an internal deletion. One survivor, no. 6 in Fig. 3, exhibited a different type of change – an extra band located between the typical 108.7- and 132.8-kb NotI bands. An additional band is also visible after digestion of the survivor no. 6 genome with SpeI restriction enzyme (Fig. 4). Such DNA gain without a loss of any band might be due to a duplication event encom-

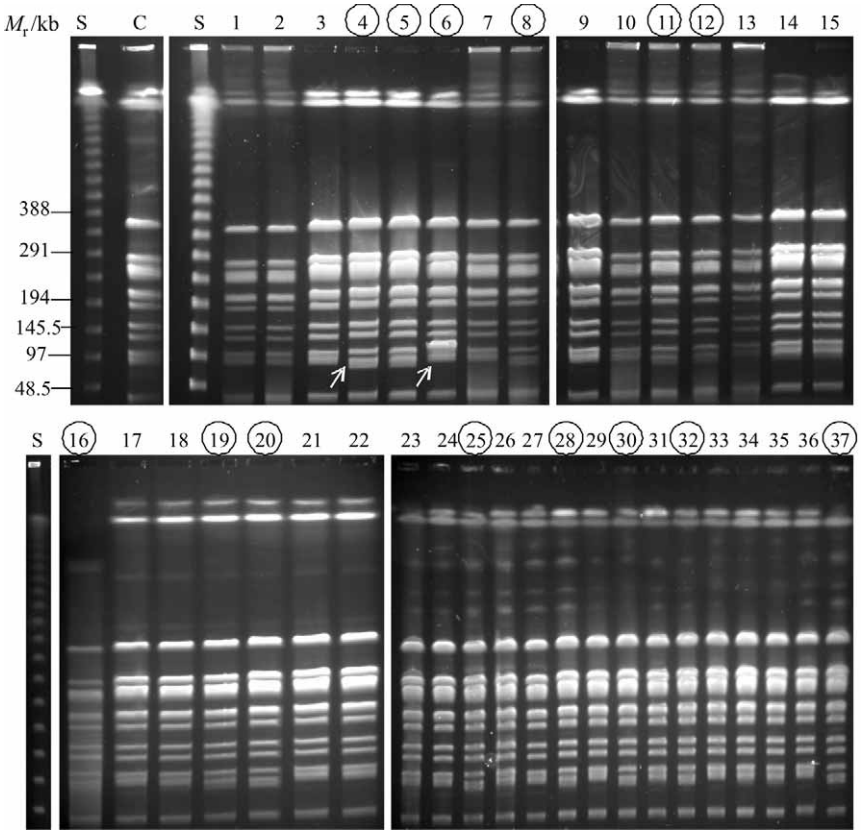

Fig. 3. PFGE analysis of NotI-treated DNA from 37 *E. coli* wild-type survivors of 3.2 kGy γ-irradiation. S=λ DNA ladder as a molecular mass standard, C=DNA of unirradiated (control) cells. Rearranged DNA patterns are marked with circles. Arrows indicate two types of DNA rearrangements

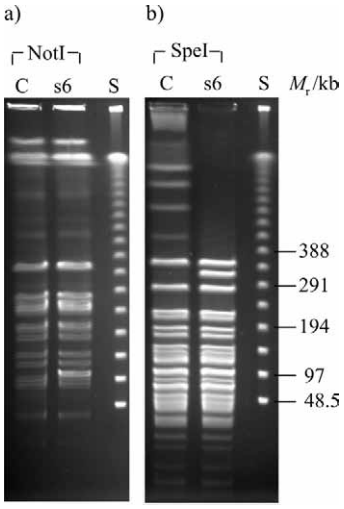

Fig. 4. PFGE analysis of DNA from wild-type *E. coli* digested with: a) NotI and b) SpeI restriction enzymes. C=DNA of unirradiated (control) cells, s6=DNA of the survivor of 3.2 kGy no. 6 (see Fig. 3), S=λ DNA ladder as a molecular mass standard

Table 2. γ-Sensitivity test for *E. coli* wild-type survivors isolated after irradiation with 3.2 kGy

Survivor[a]	Relative survival at 2 kGy[b]	Survivor[a]	Relative survival at 2 kGy[b]
Control	1.00	Control	1.00
No. 6 (R)	0.42	No. 30 (R)	0.87
No. 23	18.47	No. 31	5.05
No. 24	1.26	No. 32 (R)	3.98
No. 25 (R)	8.49	No. 33	1.19
No. 26	9.26	No. 34	3.94
No. 27	8.30	No. 35	0.60
No. 28 (R)	1.51	No. 36	0.35
No. 29	1.62	No. 37 (R)	13.21

[a]Survivors are marked with the same number designations as in Fig. 3. The unirradiated wild-type culture was used as a control. Cultures of survivors that exhibited rearranged DNA pattern are marked with (R)
[b]Survival after 2-kGy γ-irradiation of each survivor was given relative to that of the unirradiated (control) culture. Values are the average of three measurements for each culture

passing a restriction site. In a control experiment, in which the DNA of the unirradiated cells (18 samples) and the DNA of the cells irradiated with a lower dose (480 Gy) of γ-radiation (16 samples) were analyzed, we found no genome rearrangements (data not shown).

Origin of genome rearrangements in γ-irradiated wild-type cells

Hypothetically, it is possible that genome rearrangements detected in heavily irradiated wild-type cells did not arise as a consequence of DNA repair after γ-radiation, but instead that they appeared as a consequence of selection of radiation-resistant mutants existing in the population prior to irradiation. Therefore, we regrew some of 3.2 kGy survivors and tested them for γ-resistance phenotype by measuring their survival after 2 kGy of γ-radiation. Most of the tested survivors exhibited γ-resistance similar to that of the unirradiated control (Table 2). Five survivors (nos. 23, 25, 26, 27 and 37) show approx. 10-fold increase in γ-resistance but only two of them (nos. 25 and 37) are characterized by rearranged genome (Table 2 and Fig. 3). The obtained results do not reveal significant correlation between gross DNA rearrangements and increased γ-resistance. This suggests that genome rearrangements in γ-irradiated *E. coli* cells originate from postirradiation DNA repair rather than being selected by radiation.

The identical change in NotI patterns of 13 survivors (Fig. 3) seems to result from a deletion within the 98.6-kb fragment. We examined this fragment for DNA motifs that could promote deletion events, and identified e14 prophage located within the fragment. It had previously been reported that exposure to UV- and γ-irradiation, as well as other treatments that induce bacterial SOS-response, facilitated excision of e14 prophage from *E. coli* chromosome (*36*,*39*). This prophage is 15.2 kb long and its removal would cause a shift of 98.6-kb fragment similar to the shift that we observed in the changed NotI pattern of 13 survivors (Fig. 3). To check whether this change corresponds to e14 prophage excision, we conducted a PCR test on the DNA of two survivors (nos. 32 and 37) carrying a deletion of the 98.6-kb fragment. Fig. 5 shows the results of PCR tests confirming that these survivors have indeed lost the e14 prophage. Interestingly, while the PCR on unirradiated control cells shows a strong signal corresponding to the presence of e14, a low intensity signal corresponding to the e14 absence is also detectable (Fig. 5b). Therefore, it seems that a small fraction of wild-type cells has spontaneously excised e14.

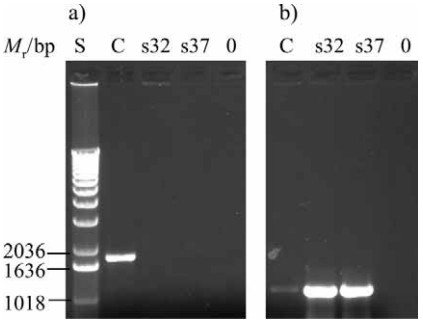

Fig. 5. PCR reactions using different combinations of primers confirm the: a) presence or b) absence of e14 prophage in the *E. coli* genome. C=unirradiated wild-type cells as a control for the presence of e14, s32=wild-type survivor of 3.2 kGy no. 32, s37= wild-type survivor of 3.2 kGy no. 37 (the same survivor numbers as in Fig. 3), 0=negative PCR control, S=1-kb DNA ladder as a molecular mass standard

We sequenced the PCR products obtained by primers e14 left and e14 right from the wild-type *E. coli* and from several e14-lacking survivors (samples no. 16, 19, 25, 28, 30, 32 and 37 in Fig. 3). Sequences obtained by both left and right primers spanned the region of e14 excision (Fig. 2). The multiple alignment of the sequenced PCR products showed no variation in the patterns of prophage e14 loss. The implication of this find-

ing is that all detected e14-lacking survivors lost the prophage by the same mechanism that is in accord with the prophage self-excision.

Survivor no. 6 is the only survivor showing a different type of gross genome rearrangements (see Figs. 3 and 4). In addition, this survivor proved to be slightly more sensitive to γ-radiation than the control (Table 2), which might be a consequence of its mutated genome.

Accuracy of genome reassembly after γ-irradiation in recA recBCD and recA recBCD sbcB mutants of E. coli

The *E. coli recA* mutants are deficient in HR and hence, in the main DSB repair mechanism (*13*). In concert with this, the newly constructed *recA recBCD* and *recA recBCD sbcB* strains proved to be much more sensitive to γ-irradiation than the wild-type cells (Fig. 1b). The result obtained with the *recA recBCD* mutant is essentialy in accord with previously published data (*40*). The *recA recBCD sbcB* strain proved to be slightly more resistant to γ-irradiation than its *sbcB*$^+$ counterpart (Fig. 1b). To test the accuracy of genome reassembly in these mutants, we chose the γ-doses of 360 and 480 Gy for the subsequent isolation of survivors, as these doses caused 10^6- to 10^7-fold reduction in the survival of *recA recBCD* and *recA recBCD sbcB* mutants, respectively. In the particular experiments from which the survivors were collected (presented in Fig. 1b), the survival of the *recA recBCD* mutant was reduced from $1.5{\cdot}10^8$ to $1.1{\cdot}10^2$ CFU/mL, and that of the *recA recBCD sbcB* mutant was reduced from $1.9{\cdot}10^8$ to $8.6{\cdot}10^1$ CFU/mL.

A total of 39 *recA* γ-survivors were examined for their DNA NotI restriction patterns: 14 *recA recBCD* survivors of 360 Gy and 25 *recA recBCD sbcB* survivors of 480 Gy. We found no changes in NotI patterns of the survivors of both mutants (Fig. 6), suggesting a high capacity for accurate DSB repair in the absence of RecA--dependent homologous recombination.

The *recA* mutants used in this work were irradiated with relatively low doses of γ-radiation that cause relatively low number of DSBs; at the dose of 360 Gy used for the *recA recBCD* cells, the extrapolated number of DSBs per cell was 33 (see Table 3; *25,41–48*). This is almost 10-fold lower number of DSBs than that inflicted on the wild-type strain by 3200 Gy. We therefore considered a possibility that some cells of the irradiated *recA recBCD* population simply evaded DSBs that could produce chromosome rearrangements. Since the probability of the DSB occurrence after irradiation follows the Poisson distribution, the spread around the average rate of occurrence (λ=33) can be calculated. The probability of observing x events is then given by $P(x)=(e^{-\lambda}\cdot\lambda^{x})/x!$ making the probability for zero DSBs $4.66{\cdot}10^{-15}$, for one DSB $1.54{\cdot}10^{-13}$, for two DSBs $2.54{\cdot}10^{-12}$, *etc*. Since the sample of the *recA recBCD* mutant exposed to radiation

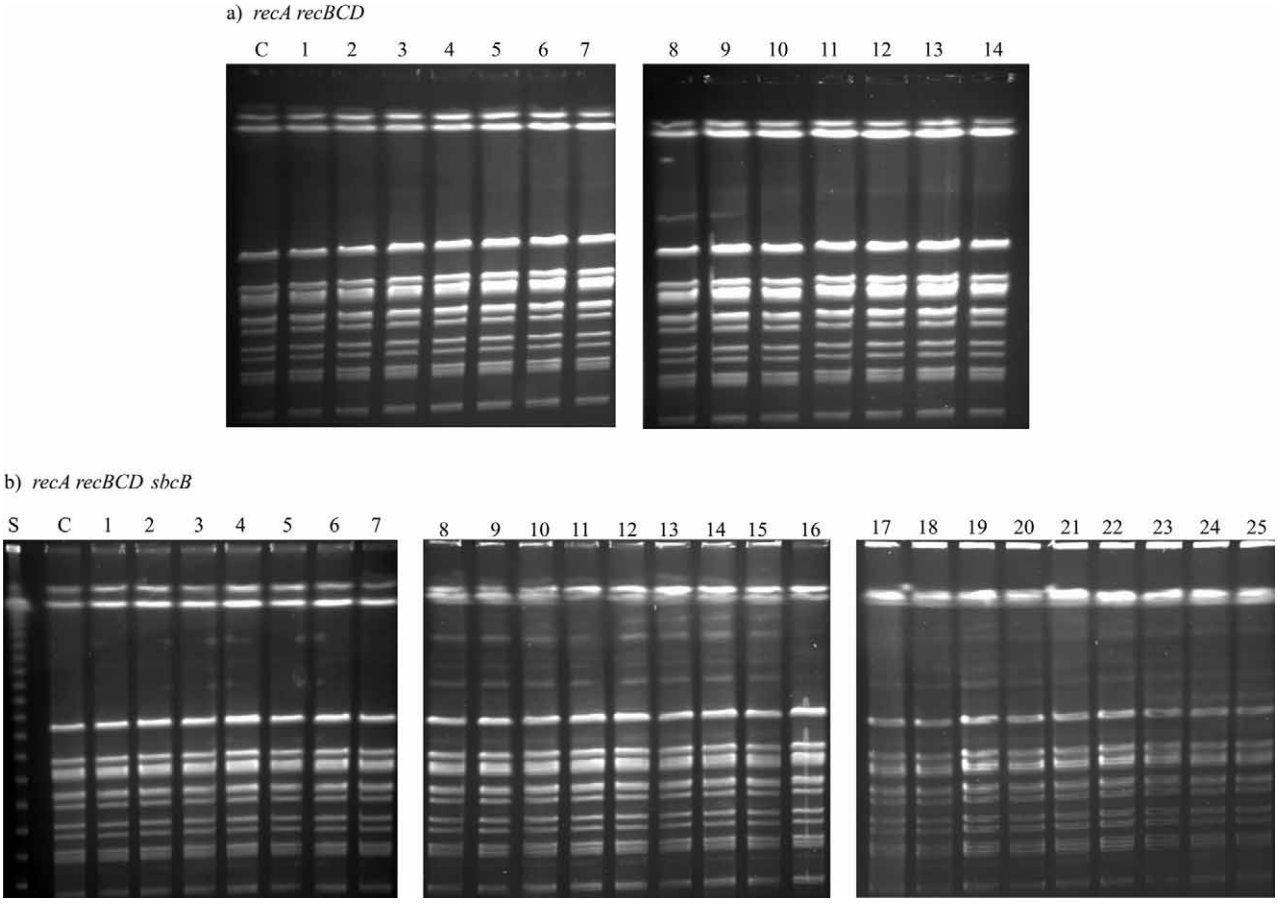

Fig. 6. PFGE analysis of NotI-treated DNA from: a) 14 *recA recBCD* survivors of 360 Gy and b) 25 *recA recBCD sbcB* survivors of 480 Gy. C=DNA of unirradiated (control) cells, S=λ DNA ladder as a molecular mass standard

Table 3. Comparison of the number of double-strand DNA breaks (DSBs) in our experimental system

Bacteria	Genome size bp	Genomes per exponentially growing cell[a]	DSBs per exponentially growing cell after 3.2 kGy for *E. coli* and 25 kGy for *D. radiodurans*[b,c]	DSBs per exponentially growing cell after 360 Gy[b,c,d]	DSBs per exponentially growing cell after 480 Gy[b,c,d]
E. coli	4639221	4–5	297	33	45
D. radiodurans	3284156	8–10	3284		

[a]Data taken from Cox and Battista (*25*)

[b]Calculated with the value of 0.005 DSB/Gy/Mbp, which is the average number of DSBs assessed in both *E. coli* and *D. radiodurans* by different techniques: sucrose gradient centrifugation (*41–44*), PFGE (*45,46*) and optical mapping (*47*)

[c]Conservative estimation for DSB number per exponential cell (the smallest number of genomes per cell was used for the calculation)

[d]Dividing wild-type and *recA recBC* cells of *E. coli* that have similar DNA content (*48*)

contained $1.5 \cdot 10^8$ cells, it was extremely improbable that any cell escaped one or more DSBs. This suggests that *recA recBCD* cells that grew into a colony after irradiation were genuine survivors, which were able to repair certain number of DSBs without producing gross genome rearrangements. This would not be surprising given that *E. coli* possesses the RecA-independent mechanisms of repair/recombination (*49,50*). However, since the exponentially growing cells are supposed to have an amount of DNA that is equivalent to more than one genome copy/cell (*48,51*), we cannot exclude the possibility that some of the *recA* cells survived due to multiple genome copies (some of which remained undamaged), rather than to DNA repair.

Discussion

γ-Radiation causes various lesions in the DNA, most severe of which are the DSBs. Accurate and efficient repair of DSBs is thought to be extremely important for the survival after γ-irradiation in the radiation-resistant bacterium *D. radiodurans*. Indeed, we have previously detected no gross genome rearrangements in a sample of 15 *D. radiodurans* wild-type survivors of 5 kGy (*16*), a dose that causes 525 DSBs per exponentially grown *D. radiodurans* cell (calculated with the rate of DSB formation from Daly (*26*)).

We have attempted to compare the frequency of gross genome rearrangements after γ-irradiation between *E. coli* and *D. radiodurans*. The major factor that influences the rearrangement frequency is the capacity of DNA repair machinery to accurately recover continuous DNA molecule after γ-irradiation. This accuracy was the subject of our analysis. However, there are also other important influences on the rearrangement frequency that need to be taken into account: (*i*) total levels of protein oxidation (carbonylation) damage, (*ii*) quantity of repeats in the genome and (*iii*) total number of DSBs per cell. In the present study, we have avoided the influence of protein oxidation by comparing the frequency of rearrangements between *E. coli* and *D. radiodurans* at the same survival dose, which corresponds to the same level of protein oxidation (*6*). The amount of repetitive sequences is comparable in both genomes (*24*), as is the rate of DSB formation (*25,26*). The total number of DSBs, however, differs between *E. coli* and *D. radiodurans* because of the different doses of γ-radiation used and because of the different DNA content of the two organisms (Table 3). This fact must be taken into account when comparing the rearrangement frequencies in these two bacteria.

The frequency of gross genome rearrangements in γ-irradiated *D. radiodurans* used in this comparison was published previously (*16*) and was obtained by the same experimental protocol as in this study. Five out of 22 (*i.e.* 23 %) *D. radiodurans* wild-type survivors of 25 kGy were found to have gross genome rearrangements (*16*). In this work, among 37 *E. coli* wild-type survivors of 3.2 kGy tested, we have detected 14 samples with rearranged genome (Fig. 3). Seemingly, this is a higher rearrangement frequency than that observed in the wild-type *D. radiodurans* (38 *vs*. 23 %). However, most of the rearrangements that we detected in *E. coli* show NotI pattern consistent with the excision of the defective prophage e14, as shown previously (*36*). Hypothetically, the prophage excision might be a consequence either of the action of e14 excision proteins or of the cellular DNA repair machinery. Rearrangement events that are a consequence of prophage's own excision proteins are not of interest if our goal is to measure accuracy of DSB repair in *E. coli*. Therefore, it would be useful to distinguish between the two ways of prophage loss – excision by the *E. coli*-encoded proteins *vs*. excision by the prophage-encoded proteins. The e14 prophage, although defective, is capable of excising itself from bacterial DNA by site-specific recombination (*39,52*). This excision occurs in the course of the cellular SOS response (*39,52*), which is expected to be induced after γ-radiation. The dependence of e14 excision on SOS induction is not yet fully understood. It was shown that e14 excision occurs in *E. coli recA441* mutants that display constitutive RecA co-protease activity which cleaves the LexA repressor and induces permanent SOS response in the absence of any insult to DNA (*39*). The RecA co-protease is also required to cleave repressor of lambda prophage, thus inducing prophage excision (*53*). It is possible that a similar mechanism could mediate excision of e14, which itself belongs to the group of lambdoid (pro)phages (*39*). Consistent with this, a recent bioinformatic analysis of the e14 sequence has revealed a gene whose product resembles the lambda repressor (*37*). Therefore, it is quite probable that e14 excision is not related to the cellular DNA repair process but is rather an internal prophage function that depends

on specific regulatory activities (such as RecA co-protease) induced upon DNA damage.

An alternative scenario for e14 excision could involve recombination events that occur independently of prophage own excisionases. For example, repetitive elements situated near e14 might cause consistent deletion events executed solely by the cellular recombinases. However, we found no exact repeats of 30 bp or longer that would be shared by the two prophage bordering regions. The lack of such repeats points away from the possibility that the cell reparatory mechanism is 'responsible' for the excision. This holds at least for the deletion events that could result from homologous recombination, a type of recombination that requires long stretches of homology, and which is expected to be dominant in the wild-type cells. Recent studies have revealed that *E. coli* possesses a RecA-independent DNA repair mechanism, termed alternative end-joining (A-EJ), which uses microhomologies (3–8 bp) to directly connect the ends of a DSB (*49*). In comparison with homologous recombination, A-EJ is an extremely inefficient and unfaithful mechanism that most often produces deletions of various sizes at sites of DSB repair (*49*). Our results do not reveal sequence modifications at the junction site that could suggest an involvement of A-EJ in e14 excision. Quite opposite, both uniformity of PFGE patterns of e14-lacking survivors and sequence analyses of e14 deletion sites indicate that the prophage loss was achieved by a precise site-specific excision. For this reason, the e14 deletion should not be considered genome rearrangement in a strict sense.

Genome rearrangements can spontaneously arise in normally growing bacterial populations, although with a low frequency (*54*). We detected no rearrangements in PFGE analysis of 18 unirradiated samples of the wild-type *E. coli* (data not shown), demonstrating low frequency of spontaneous genome rearrangements in this bacterium. A spontaneous genome change might hypothetically be associated with a change in γ-resistance phenotype. Therefore, γ-radiation treatment might cause a selection of more resistant spontaneous mutants, whose rearrangements might not be the consequence of γ-radiation. This is not the case for the rearranged survivor no. 6, whose radiation resistance is not enhanced (Table 2), but it might be the case for the e14 deletion survivors since the excision of e14 prophage has previously been linked to a slight improvement in γ-radiation resistance (*36*). It was reported that *E. coli* strains lacking e14 display approximately eightfold increase of resistance to γ-radiation, at a dose of 3000 Gy (*36*). Although being rather modest, such increase of resistence could be relevant for the selection of survivors at doses which bring a population close to extinction. The possibility for such scenario was strenghtened by our finding that unirradiated wild-type population contained a certain number of cells that lacked e14. However, we have not found a strict correlation between the increased γ-radiation resistance and the absence of e14 (survivors no. 25, 28, 30, 32, and 37 in Table 2). Although some of e14-lacking survivors (nos. 25, 32, and 37) exhibited an increased resistance to radiation, some (nos. 28 and 30) still retained nearly wild-type level of resistance. This fluctuation of γ-radiation resistance argues against the possibility that high incidence of e14-lacking survivors is a simple consequence of a selection of preexisting e14-lacking radiation-resistant clones. However, we cannot rule out the possibility that e14-lacking survivors are of mixed origin; some being selected by irradiation, whereas others being produced by irradiation.

Excision of e14 is expected to be elevated by γ-radiation-induced SOS response. The rate of its excision has been detected to be as high as 13 % at high doses of DNA-damaging agent mitomycin C (*55*). Even higher rate of excision has been detected in the *recA441* mutants during prolonged growth under conditions which derepress the host SOS response (*39*). In our study, sixteen *E. coli* wild-type survivors of 480 Gy, a relatively low dose of γ-radiation, did not show e14 excision patterns (data not shown). A high number of detected e14 deletions in survivors of 3.2 kGy (13 out of 37) could be (at least partly) a consequence of the increased excision rate of the prophage at conditions of fully derepressed and long-lasting SOS response.

If we exclude the e14-lacking strains from the category of rearranged survivors, only one out of 37 (2.7 %) survivors has been inaccurately repaired after γ-irradiation (survivor no. 6 in Fig. 3). If assumed that each DSB carries equal risk of DNA rearrangement, the frequency of rearrangements detected for *D. radiodurans* (22.7 %, *16*) and for *E. coli* (2.7 %, this paper) can be normalized by the number of DSBs per cell and then compared. When using the number of DSBs per cell from Table 3, the numbers of 0.000069 for *D. radiodurans* and 0.000091 for *E. coli* are obtained, representing the number of genome rearrangement events per DSB. Therefore, the data obtained in this work suggest that rearrangement frequencies after γ-irradiation do not differ considerably between *D. radiodurans* and *E. coli*.

In the absence of the RecA protein, γ-resistance of *E. coli* falls dramatically. This is a consequence of inactivation of the main mechanism of DSB repair in bacteria, the RecA-dependent homologous recombination. HR is a process that uses excess genome copies as a source of information for accurate DSB repair. The dependence on longer stretches of homology of the identification of intact DNA copies that serve as a guide for repair makes HR an accurate mechanism of DSB repair, vulnerable only to longer DNA repeats. The RecA-independent repair of DSBs that has been detected in *E. coli* (*49*,*50*) is limited by exonucleases (*50*) and is sometimes associated with the use of microhomologies (*49*,*50*). These repair mechanisms are less accurate than HR, because of their less stringent use of homology for reattachment of DSB ends. In *D. radiodurans*, RecA-independent DSB repair is inaccurate and leads to genome instability and gross genome rearrangements, both during normal growth and after γ-irradiation (*16*). We, however, detected no gross genome rearrangements in the 39 γ-irradiation survivors of *E. coli recA* strains (Fig. 6). High sensitivity of these strains to γ-radiation limited us to the use of relatively low doses of γ-radiation, causing a low number of DSBs. Still, the doses of 360 and 480 Gy cause 33 and 45 DSBs per *E. coli* cell (Table 3), respectively, and only one DSB left unrepaired can lead to cell death. Our results sug-

gest that surviving *recA* cells were able to repair these breaks without generating large genome rearrangements, demonstrating a surprisingly high accuracy of RecA-independent DSB repair in *E. coli*. This, however, must be taken with reserve, because small DNA rearrangements cannot be detected in our system. Also, since the doses applied on *recA* mutants cause relatively small number of DSBs in exponentially growing cells (Table 3), it is statistically possible for some cells to evade large number of DSBs, consequently increasing their survival and decreasing the probability for genome rearrangements. On the other hand, it is known that γ-radiation frequently produces single-strand breaks (SSBs) whose number is approx. 20-fold higher than the number of DSBs generated by the same dose of radiation (*56*). In exponentially growing *E. coli* cells, SSBs may cause secondary DSBs associated with the collapse of replication forks (*57*). Part of secondary DSBs may also arise from the attempted base-excision repair of radiation-induced clustered DNA lesions (*58*). Hence, the actual number of the DSBs that arise during postirradiation growth could be significantly higher than that measured immediately after irradiation.

Another complication when estimating the effect of ionizing radiation on the *recA* cell survival is associated with viability of unirradiated cells. In exponentially growing culture of the *recA recBC(D)* mutant, less than 20 % of cells is viable (*i.e.* capable of forming a colony on solid medium), whereas the rest of the population comprises residually dividing and nondividing cells (*31*,*48*). High mortality of *recA recBCD* mutants is largely associated with the accumulation of SSBs and DSBs that arise spontaneously during DNA replication (*48*,*59*). Hence, DNA content of viable *recA recBCD* cells may often consist of both functional and nonfunctional genome copies. Conceivably, in such circumstances, multiple genome copies cannot contribute to the passive radiation resistance as they do in the wild-type cells.

Our results do not reveal any significant correlation between gross genome rearangements and resistance to γ-radiation in *E. coli*. However, they show that acute exposure to a high dose of γ-radiation may lead to the formation and/or selection of bacterial clones with increased radiation resistance. These results add to several previous studies demonstrating a remarkable capability of bacteria for rapid adaptation to ionizing radiation (*16*, *36*,*60*,*61*). This should be taken into account when considering the strategies for exploiting ionizing radiation in food processing.

Conclusion

Escherichia coli shows surprisingly high accuracy of genome reassembly after γ-irradiation. Our data do not suggest a considerable difference between frequencies of gross genome rearrangements in wild-type *E. coli* and *D. radiodurans* after γ-irradiation. Hence, it seems that higher sensitivity of *E. coli* to γ-radiation is not due to less accurate repair of damaged DNA. The postulated efficiency of DSB repair in γ-radiation resistant organisms such as *D. radiodurans* might be manifested in features other than the ability to avoid genome rearrangements. It is possible that radiation-resistant organisms possess some mechanistic specificities that allow them to recover their genome from shorter DNA fragments (note that the average size of DNA fragments in *D. radiodurans* after 25 kGy is approx. 8 kb whereas that in *E. coli* after 3.2 kGy is approx. 62 kb, as calculated from the data presented in Table 3). Also, the speed of genome reassembly could be higher in radiation-resistant organisms, which might have a significant impact on cell survival.

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