J. Microbiol. Biotechnol. (2010), **20**(1), 30–38
doi: 10.4014/jmb.0907.07005
First published online 12 October 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Unbalanced Restriction Impairs SOS-induced DNA Repair Effects

Katna, Anna, Robert Boratynski, Beata Furmanek-Blaszk, Natalia Zolcinska, and Marian Sektas*

Department of Microbiology, University of Gdansk, 80-822 Gdansk, Kladki 24, Poland

Received: July 3, 2009 / Accepted: August 5, 2009

The contribution of a type II restriction–modification system (R-M system) to genome integrity and cell viability was investigated. We established experimental conditions that enabled the achievement of hemimethylated and unmethylated states for the specific bases of the recognition sequences of the host's DNA. To achieve this, we constructed the MboII R-M system containing only one (*i.e.*, M2.MboII) out of two functional MboII methyltransferases found in *Moraxella bovis*. Using the incomplete R-M system, we were able to perturb the balance between methylation and restriction in an inducible manner. We demonstrate that upon the SOS-induced DNA repair in mitomycin C treated cells, restriction significantly reduces cell viability. Similar results for the well-studied wild-type EcoRI R-M system, expressed constitutively in *Escherichia coli*, were obtained. Our data provide further insights into the benefits and disadvantages of maintaining of a type II R-M system, highlighting its impact on host cell fitness.

Keywords: Restriction endonuclease, DNA methyltransferase, type II R-M systems, SOS induction

Restriction-modification (R-M) systems have been widely recognized as the major defense force in the bacterial protection system directed against foreign DNA invasion [49]. They are present in almost all bacterial species [42]. There is no doubt that the balance between cellular levels of R and M activities ought to be precisely regulated so as not to affect the viability of the bacterial cell [3, 23, 24, 45–47]. Interestingly, there are many examples of bacteria abundant in various R-M gene complexes in their own genomes [42]. Therefore, very reliable regulatory mechanisms for gene expression ensuring their stable maintenance are required [36]. Such functional balance is supported by very efficient recombinational and repair mechanisms [3, 13, 19, 23, 24]. It is perhaps reasonable to believe that the death of a particular cell is more likely to happen in the case of overproduction of the endonuclease in many artificially constructed systems. Interestingly, similar lethal effects can be observed after a decrease in an R-M enzyme's activities as a result of the loss of its genetic determinants. Apart from the last case, which has been well described [28], the impact of all other kinds of R-M-mediated molecular events, which could participate in genome organization and stability, remain poorly characterized and still obscure. It seems reasonable to suggest that there are several circumstances when restriction endonucleases (REase) may act on the host genome: (1) in the case of the loss of R-M genes, when gradually decreased modification activity leads to autorestriction cleavage of the chromosome, which is generally lethal for the majority of cells, although some of them might be rescued by repair mechanisms [19, 20, 37]; (2) during the period of self-establishment of various R-M systems into a new host [35, 38], which would also include their different adaptation times [39]; and (3) in the case of the regaining of full functionality by a formerly defective R-M system, by acquisition of the cognate REase gene to pair with the resident methyltransferase (MTase) specificity [1, 2, 30, 39]. To date, the last two cases have been poorly investigated because of difficulties in assaying them biochemically and genetically. This might prove interesting, especially in the context of those R-M systems that consist of a restriction REase in tandem with two MTases genes coding for two independent specificities [42]. It has been shown that the level of activity, and even specificity, for two existing MTases can be strikingly different, one from the other [8, 16, 25, 33]. The MboII R-M system from *Moraxella bovis* represents one of the most interesting systems from the functional point of view. In contrast to common type II R-M systems, it consists of three genes coding the R.MboII restriction endonuclease, and two separate DNA MTases, M1.MboII (recognizing the 5'-GAAGA-3' sequence [32]) and M2.MboII (specific to 5'-TCTTC-3' [16], a strand complement of the canonical site).

*Corresponding author
Phone: +48-58-523-63-98; Fax: +48-58-523-64-20;
E-mail: sektas@biotech.ug.gda.pl

In the present paper, we describe another aspect arising from the maintenance of the R-M systems, which adds to our understanding of the molecular basis of bacterial genetic stability. Apparently, there are several critical moments when some regions of the chromosome may become vulnerable to residual REase activity. It includes, for instance, appearances of hemi- or nonmethylated recognition sites during the propagation of replication forks or the repair processes of the damaged regions of DNA [3]. We applied the MboII R-M system to examine our hypothesis of interference of restriction to DNA repair by means of excision repair and/or homologous recombination. An intrinsic feature of the unbalanced MboII R-M system used here is the inducible disturbance in equilibrium between protective methylation and restriction. Specifically, owing to the properties of M2.MboII [16], it is not possible for one complimentary strand of each canonical sequence to be methylated (hemimethylation), giving rise to the temporary occurrence of completely unprotected sites on the newly repaired genome regions triggered by mitomycin C (MMC) damage.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Escherichia coli (*E. coli*) HB101 F^- Δ (*gpt-proA*) *62 leuB6 supE44 ara-14 galK2 lacY1* Δ (*mcrC-hsdRMS-mrr*) *rpsL20* (Str^R) *xyl-5 mtl-1 recA13* [7] was used as the cloning host. The SOS reporter strain *E. coli* ER1992 [F^- Δ (*argF-lac*) *U169 supE44 e14*$^-$ *dinD1*::*Mu dI1734* (Km^R, $LacZ^+$) *rfbD1? relA1? endA1 spoT1? thi-1* Δ (*mcrC-mrr*) *114*::IS*10*] [15] and MG1655 [4] were used in most experiments. *E. coli* cells were grown in Luria–Bertani (LB) broth or agar [34] with antibiotics (Sigma) at the following concentrations: ampicillin (Amp), *100* μg/ml; chloramphenicol (Cam), 25 μg/ml; kanamycin (Kan), 15 μg/ml; and tetracycline (Tet) 15 μg/ml. L-Arabinose (Sigma) was used at 0.2%, and IPTG (Sigma) and mitomycin C (MMC, Sigma) were as indicated in the experiments. The plasmids used in this work (Table 1) were introduced into the cells by a standard chemical transformation procedure [43].

Construction of Bacterial Strains

To construct the *E. coli* strain expressing *mboIIM2* and *mboIIM1* genes from the arabinose-dependent P_{araBAD} promoter, the *mboIIM1* gene was cut out from pMboM.1 [5] by PstI digestion and put into the PstI site of the pBAD24 vector [17]. Next, the EcoRI–SalI

Table 1. Plasmids.

Plasmid	Description	Source or reference
pACYC184	Cloning vector with P15A *ori*, Cam^R, Tet^R	[9]
pACYCeco	3.6-kb HincII DNA fragment carrying EcoRI R-M system from pSCC2 plasmid inserted into BsaBI site of pACYC184, Cam^R, Tet^R	This work
pACYCphoA	1.5-kb PCR-amplified promoterless *phoA* gene inserted between HindIII and BamHI fused to a P_{tetA} promoter of pACYC184, Cam^R, Tet^R	This work
pANTS	pBR322 replicon, integrative vector with λ *attP* site and NotI-excisable *ori* region, Amp^R	[40]
pANTSeco	6.0-kb ScaI–XbaI fragment carrying *tetA-ecoRIR-ecoRIM* from pACYCeco in pANTS, Amp^R, Tet^R	This work
pANTSeco-R	pANTSeco with frameshift mutation in *ecoRIR* gene	This work
pANTSmboBAra	3.6-kb ClaI–HindIII fragment containing *araC*-P_{araBAD}::*mboIIM.2 mboIIM.1* cassette from pBADmboBA in pANTS, Amp^R	This work
pANTSphoA	1.9-kb SalI–XbaI fragment containing *phoA* gene from pACYCphoA in pANTS	This work
pBAD24	Arabinose-inducible expression vector with *araC*-P_{araBAD}	[17]
pBADmboBA	1.1-kb EcoRI–SalI fragment containing *mboIIM.2* and 1.1-kb PstI fragment carrying *mboIIM.1* gene under L-arabinose control in pBAD24	This work
pBR322	Cloning vector, Amp^R, Tet^R	[6]
pBRmboIIMB.20	1.1-kb Fusion gene P_{tetA}::$P_{mboIIM2}$::*mboIIM2* in pBR322, Amp^R	[16]
pINTtsCm	λ Int-delivery pSC101Ts replicon containing thermoinducible expression cassette cI857-P_R::*int*, Cam^R	[21]
pMboM1.1	1.1-kb Fragment DNA containing the *mboIIM.1* gene in pUC8, Amp^R	[5]
pMboR3.0	pACYC184 replicon with 1.3-kb fragment DNA containing P_{lac}::*mboIIR* under $lacI^q$ control, Tet^R	[5]
pMboRMB.7	1.4-kb PvuII P_{lac}::$P_{mboIIM2}$::*mboIIM2* cassette from pUCMB.3 inserted in ScaI site of pMboR3.0, upstream of the *mboIIR* gene in antiparallel orientation, Tet^R. In this work, the plasmid constitutes the MboII $r^+m.2^+$ system.	This work
pSC101	pSC101 replicon, Tet^R	[11]
pSCC2	pSC101 plasmid carrying EcoRI R-M system, Amp^R	[10]
pUC18	Cloning vector, Amp^R	[50]
pUCMB.3	1.1-kb BamHI *mboIIM2* cassette from pBRmboIIMB.20 in pUC18	[16]

fragment carrying the *mboIIM2* gene from pUCMB.3 [16] was inserted into the freshly constructed pBAD24mboA. Then, a ClaI–HindIII cassette containing *araC*-P_{araBAD}-*mboIIM2-mboIIM1* was subcloned into the pANTS integration plasmid, containing a λ-phage *attP* sequence [40]. To insert the DNA cassette into the *E. coli* ER1992 chromosome [15], suicide plasmid pANTSmboBAra was constructed by removal of ori_{pBR} by NotI digestion, followed by agarose-gel purification and religation (2 h incubation at room temperature with T4 DNA ligase; Epicenter). Next, Int/*att* recombination was employed, using Int-delivery temperature-sensitive plasmid pINTtsCm [21], to introduce the Δ*ori* suicide vector into the *attB* insertion site of the chromosome. The resulting recombinant strain was named ER1992mboBAra [λ*attB::bla* (Amp^R) *araC*-P_{araBAD}-*mboIIM2-mboIIM1*].

To construct an *E. coli* ER1992 host with a chromosomally encoded EcoRI R-M system, EcoRI R-M genes were cut out with HincII from the pSCC2 plasmid [10], subcloned into the BsaBI site of pACYC184, and named pACYCeco. Next, the ScaI–XbaI blunted-DNA fragment coding for tetracycline resistance (*tetA*$^+$) and EcoRI R-M genes were subcloned into the SmaI site of a pANTS integrative vector [40]. An Int/*att*-mediated integration method was employed [21] to insert Δ*ori* pANTSeco into the *attB* locus in the ER1992 chromosome. The resulting strain was named ER1992E. As a restriction-deficient control, an isogenic *ecoRIR*$^-$ strain (ER1992EΔR) was constructed. The endonuclease gene was inactivated by BglII digestion of the pANTSeco, followed by the filling-in of the DNA ends by the Klenow polymerase I DNA and religation. As a result of this, the pANTSeco-R vector experienced a shift in the *ecoRIR* reading frame after the 65th codon, generating a premature stop codon (TAG) after the 77th codon. pANTSeco-R was integrated with the ER1992 chromosome. To compare the viability of the ER1992 host with its derivatives carrying R-M systems under MMC exposure, the relative viabilities of particular cultures grown at 37°C for 16 h with or without MMC treatment (0.75 μM) were calculated as a proportion between c.f.u. values measured in treated and untreated cultures ($c.f.u._{MMC}/c.f.u._0$) at the same points of growth time.

Plasmid Constructions

To construct a plasmid carrying a functionally unbalanced MboII R-M system composed of an endonuclease gene and only one methyltransferase gene (hereafter designated as the MboII $r^+m.2^+$ system), the PvuII/PvuII fragment from the pUCMB.3 plasmid bearing the *mboIIM2* methyltransferase gene in transcriptional fusion with the P_{lac} promoter was subcloned into the ScaI site of the pMboR3.0 plasmid containing the *mboIIR* gene under P_{lac} control [5]. In the resultant plasmid (pMboRMB.7), the *mboIIM2* and *mboIIR* genes are in opposite orientation and their expression depends on IPTG induction.

To construct plasmids with the P_{tetA}–*phoA* transcriptional fusion providing a measurable and reliable level of basal PhoA activity, a system for the constitutive expression of the *phoA* gene was constructed, combining the P_{tetA} promoter of pACYC184 [9] with a promoterless *E. coli* ER1992 *phoA* gene. A DNA fragment of approximately 1.5 kb containing the *phoA* gene was amplified by PCR using primers Pho5 (5'-CCAAGCTTTAATGCGGTAGTTT*CATGGAGAAAATAAA***GTG***AAACAA*-3'), containing the −10 site of P_{tetA} (in bold) and part of the *phoA* gene (in italics, start codon in bold), and Pho3 (5'-GCGGATCC TTATTTCAGCCCCAGAGCG-3'). Then, the PCR-generated product was double digested with HindIII and BamHI (underlined in primers, respectively) and ligated into the corresponding restriction sites of the vector pACYC184, resulting in pACYCphoA. The *phoA* fusion gene was then subcloned in the pANTS vector, creating pANTSphoA. Positive blue-color recombinants were selected on LB agar supplemented with appropriate antibiotic and 25 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt (Fermentas).

Mitomycin C-induced DNA Damage, and RF and IF Measurements

E. coli ER1992 bacteria bearing pMboRMB.7 were grown at 37°C in LB medium to an early-log phase, to an optical density at 600 nm (OD_{600}) of 0.12–0.15. Then, the culture (20 ml) was split and their growth was continued for 1 h with or without MMC at 0.5 μg/ml (1.5 μM) of the final concentration. Both cultures were split again into two subcultures, (i) one of them growing in preexisting conditions, and (ii) the second IPTG-treated (0.1 mM) to induce expression of the MboII $r^+m.2^+$ system for the next hour. To determine viable cell counts, the samples were taken every hour to measure c.f.u./ml by means of serial dilutions of cultures, and then 0.1-ml aliquots of the different dilutions were plated on LB-agar supplemented with Tet and/or Kan. c.f.u. were scored after overnight incubation at 37°C. Next, a so-called reduction factor (RF) was calculated at each time point; that is, the ratio of the mean value of the c.f.u./ml of the untreated sample to the mean value of the c.f.u./ml of the treated sample (with MMC and/or IPTG).

In order to measure the induction of the promoters by MMC, followed by its removal from the growth medium, the SOS-chromotest method was used [41]. For this purpose, one control (growth without MMC) and three testing cultures of ER1992 bearing both pMboRMB.7 and pANTSphoA plasmids were grown for 1 h in the presence of MMC (1.5 μM), and were then gently pelleted by centrifugation and rinsed twice with fresh LB broth medium in order to remove the MMC. All the bacterial pellets were resuspended in the starting volumes of an LB medium and allowed to grow at 37°C for 30, 60, or 90 min, respectively. The SOS induction factor (IF) for these cultures was determined. The level of activity of the reporters β-galactosidase (from *dinD1::lacZ* transcriptional fusion) and alkaline phosphatase (*phoA*) were estimated by employing ONPG or *p*-nitrophenyl phosphate as substrates, respectively. One IF is the ratio of β-galactosidase units to alkaline phosphatase units in the treated bacteria divided by the same ratio of the two enzymes units in the untreated control bacteria.

In a separate experiment, the bacteria ER1992 harboring pMboRMB.7, after 30, 60, or 90 min of recovery time, underwent 1 h IPTG induction (0.1 mM), and then RF values for all the tested cultures were determined using an IPTG-untreated culture as a control. For analysis of the chromosomal DNA degradation, 3-ml samples were taken for isolation of the whole cellular DNA [20]. DNA samples (20 μl) were electrophoresed overnight at 5 V/cm, in 0.65% SeaKem GTG agarose gel (FMC) in 0.5×Tris-borate-EDTA buffer. The DNA was observed and photographed after ethidium bromide staining.

β-Galactosidase and Alkaline Phosphatase Assays

Cells were grown at 37°C in LB with aeration. The media were supplemented with appropriate antibiotics, and with MMC or IPTG, as indicated. The cells (0.1-ml culture aliquots) were permeabolized with 25 μl of 0.1% sodium dodecyl sulfate and 50 μl of chloroform and assayed for the level of β-galactosidase produced from the fusion gene *dinD1::lacZ*$^+$, by using 0.2 ml of ONPG (4 mg/ml) as previously described [34]. To determine the alkaline phosphatase

activity level, 0.1-ml samples were mixed with 0.6 ml of buffer T (1 M Tris pH 8), and then the cells were disrupted as described above. Samples were equilibrated at 37°C and the reaction was started by addition of 0.3 ml of *p*-nitrophenyl phosphate (4 mg/ml in buffer T) until a yellow color developed, and then was stopped by the addition of 0.5 ml of 1 M Na_2HPO_4 [44].

Fluorescence Microscopy

The chromosomal DNA of *E. coli* ER1992 harboring pMboRMB.7, grown in the presence or absence of MMC or IPTG, was stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) at a concentration of 1 μg/ml. For visualization by fluorescence microscopy, 5 μl of cells was harvested at various time points and immobilized on a poly-L-lysine-treated slide coverslip. The cells were observed under 1,000× magnification using a BX51 fluorescence microscope (Olympus) and photographed with an F-View-II CCD camera (Olympus). Measurements and image analysis were conducted with AnalySIS software.

RESULTS AND DISCUSSION

Rationale for the Application of a Functionally Unbalanced MboII R-M System

In order to investigate the imbalance incidents in normally functioning R-M systems and their impact on bacterial genome integrity and cell survival, we established an experimental system that provides cellular conditions for the transient excess of restriction activity over protective methylation of the recognition sites. Such a molecular tool was created to perturb the equilibrium between restriction and modification by employing the MboII R-M system from *Moraxella bovis* ATCC 10900 [5]. An initial study of the *mboIIM2* gene expression in *E. coli* cells revealed that M2.MboII MTase, whose gene was present on the medium-copy pBR322 derivative vector, was not able to protect all the contained MboII recognition sites [16]. Thus, the MboII R-M system that is devoid of the *mboIIM1* gene was used in this work as an inducible unbalanced R-M system, named MboII $r^+m.2^+$. Since M2.MboII-mediated methylation causes SOS induction in *E. coli* wild-type strains [16], all experiments were carried out in *E. coli* deficient for McrBC and Mrr. We have constructed plasmid pMboRMB.7, which carries divergently oriented *mboIIM2* and *mboIIR* genes, both under control of the *lacZ* promoter/LacI repressor. We have assayed the functional relationship between expression of these two genes and their influence on a host cell's viability, either under non-inducing or inducing conditions. The representative data of the post-expression of the MboII $r^+m.2^+$ phenotype in the ER1992 SOS reporter strain are shown in Table 2. The SOS response, manifested both by the *dinD1::lacZ* expression and cell filamentation in this particular host, was caused by *mboIIR* and *mboIIM2* overexpression but not by expression of *mboIIM2* alone. The two features of SOS, mentioned above, suggested the appearance of DNA breaks in the chromosome structure. Under the uninduced conditions, the MboII $r^+m.2^+$ system only slightly elevated the basal level of SOS induction in comparison with the restriction-deficient ER1992 strain (Table 2). This was also confirmed by microscopic analysis of cell shape in preparations of bacteria representing both phenotypes, as well as in bacterial growth rates. Bacteria harboring the pMboRMB.7 plasmid displayed a slightly slower rate of cell mass production under non-inducing conditions (Table 2), whereas very few filamentous cells were also observed (data not shown). A significant reduction in the OD_{600} value was observed after 2 h incubation with IPTG, which induced MboII $r^+m.2^+$ expression. Depending on IPTG concentration, the SOS-induced β-galactosidase production was several times greater than the background level (Table 2). Thus, the MboII $r^+m.2^+$ system met our requirements to be unbalanced in an inducible manner.

Unbalanced Restriction Diminishes SOS-induced DNA Repair Efforts

We investigated the survival of mitomycin-C-treated bacterial cells carrying the MboII $r^+m.2^+$ system, following IPTG induction to trigger an imbalance in cellular R-M activities. We focused on the interference of restriction on the DNA repair process. Single-strand nicks and double-strand breaks may lead to unmethylated target sequences by means of recombinational repair [3]. Indeed, as it has been reported recently, the RecA/BCD- and RecFOR-mediated DNA repair pathways are required for restriction-mediated breakage of the genome [18, 19, 22]. The DNA-modification agent MMC forms a broad spectrum of products, all of which provoke an SOS response and

Table 2. Influence of the unbalanced MboII $r^+m.2^+$ system on culture growth monitored by an SOS-induced expression of the *dinD1::lacZ* gene.

E. coli ER1992[a]	OD_{600} after 2 h induction	β-Galactosidase activity (Mu)[b]
pBRmboIIMB.20 (M2.MboII$^+$)	1.09±0.04	46±15
pMboRMB.7 (MboII $r^+m.2^+$)	0.97±0.14	90±11
pMboRMB.7 with 0.1 mM IPTG	0.75±0.20	551±92

[a]The *E. coli* ER1992 culture with the MboII $r^+m.2^+$ system residing on plasmid pMboRMB.7 was split at approximately OD_{600} of 0.1–0.15, and then propagated further for 2 h, with or without IPTG inducer.

[b]Data given are the means of at least four experiments with±SD calculated in Miller units (Mu).

inducible DNA repair. These include a small percentage of interstrand cross-links between guanosines [29]. According to many reports, interstrand cross-links are repaired by the sequential efforts of combined nucleotide excision repair machinery and two subpathways of homologous recombination [14]. We assumed that this yielded, in effect, many regions of DNA with unprotected recognition sites. Computer analysis of the chromosome sequence data concerning MG1655 (parent of ER1992) revealed the presence of nearly 3,000 "extended" MboII sites 5'-CGAAGA-3'/5'-TCTTCG-3', which could be potentially interstrand cross-linked by MMC action directly, and in a favorable manner [29]. Obviously, far more numerous MboII sites that are situated in close vicinity to the cross-linked nucleotides could be displaced by excision repair and/or homologous recombination mechanisms [14].

In our experiment, the cultures of the ER1992 bacteria carrying the pMboRMB.7 plasmid were treated with MMC (1.5 μM of final concentration) for 1 h and then underwent IPTG induction (0.1 mM for 1 h) to express the *mboIIM2* and *mboIIR* genes (Fig. 1A). We evaluated the influence of MMC and/or R.MboII on cell growth inhibition and bacterial viability either as individual or combined effects. Both agents, being harmful to DNA structure, were able to induce an SOS response and cause most of the cells to become filamentous (Fig. 1B). Besides this, very long nondivided forms of the chromosome were observed, something which was not found in the case of control cells. Inhibitions of growth effected by the MMC agent alone, calculated as a reduction factor (RF) after 1 and 2 h of cultivation, were 5.6 and 10.6, respectively. Survivals of the bacteria grown under the *mboIIR* expression conditions in the control or MMC-treated culture were 4.5 and 14, respectively (Fig. 1A). Taking into account that the growth inhibition effect by the MMC agent alone by 2 h was at 10.6, the combined effect obtained for MMC and R.MboII (RF=148) is not simply additive but has all the hallmarks of a synergistic effect. It should be noted that these results were obtained from conditions in which the bacterial cells were permanently subjected to DNA damage by MMC, and therefore the DNA repair is persistently continued throughout the experiment. Hence, we decided to look at the efficiency of the repair process after the removal of the MMC agent, and further affected only by unbalanced restriction. In this case, the combined effect of the two DNA-damaging agents varied significantly upon removal of the MMC agent, depending on the time allowed for DNA repair prior to the 1 h IPTG induction of the MboII $r^+m.2^+$ system (Fig. 2A). The survival of bacteria strongly correlated with the duration of the "regenerative" growth of cells. The shorter recovery times after MMC-treatment of the R.MboII-caused restriction was more effective (RF of 16.1±6.4 versus 8.2±1.2 and 2.8±0.6 for growth by 30, 60, and 90 min, respectively). No restriction effect (RF=1.8±0.4) was observed in host cells protected by pre-expression of both MTases ($M1.MboII^+$, $M2.MboII^+$), even if the shortest time of cell recovery was applied (Fig. 2A). Cells after 1.5 h of repair time displayed an "autorestriction plateau" (RF=2.8) similar to the MMC-free control strain with *mboIIR* expression alone (RF=4.5, see Fig. 1A). These results suggested that the SOS-repair generated unmethylated DNA regions that could be the targets for unbalanced restriction activity, and that the level of DNA degradation was dependent on the accessible number of the newly created unmethylated sites. In all the subcultures studied, the process of SOS-induced repair of the DNA started at the same time. However, the protective M2.MboII methylation of newly repaired DNA regions, possible under repression conditions as a result of a leaky transcription from the P_{lac} promoter of *mboIIM2*, was most insufficient in cells allowed only 30 min for the recovery after the MMC treatment. Therefore, the accumulation of

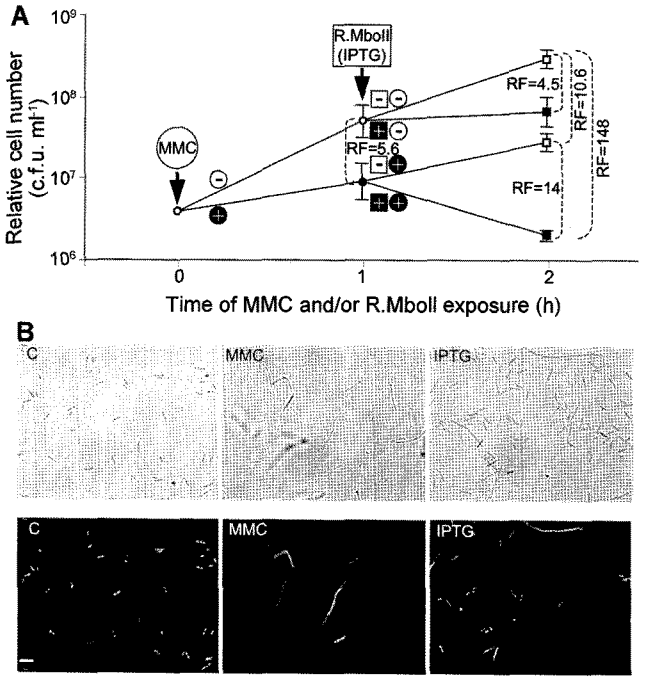

Fig. 1. Restriction interference with mitomycin-C-induced repair of the DNA.

A. Influence of mitomycin C treatment and/or MboII $r^+m.2^+$ system expression on *E. coli* ER1992/pMboRMB.7 cell survival. The initial culture was split at OD_{600} of 0.12 on two, and then, after 1 h of growth, into four independent culture lines. Time-points of MMC (1.5 μM) or IPTG (0.1 mM) addition are marked by arrows and specified as a plus or minus within a circle (black or white) or a rectangle (black or white), respectively. Error bars represent the standard deviations of three independent experiments. Reduction factor (RF) values for the cultures treated with MMC after 1 and 2 h exposure or/and after 1 h *mboIIR* expression induced by IPTG were determined. **B.** Fluorescence microscopy of cells. The ER1992/pMboRMB.7 cells, harvested after 1 h exposure either to MMC (1.5 μM) or IPTG (0.1 mM), or mock-treated (C) were fixed and stained with DAPI for visualization of cell morphology (upper row) and nucleoids (bottom row). The scale bar indicates 5 μm.

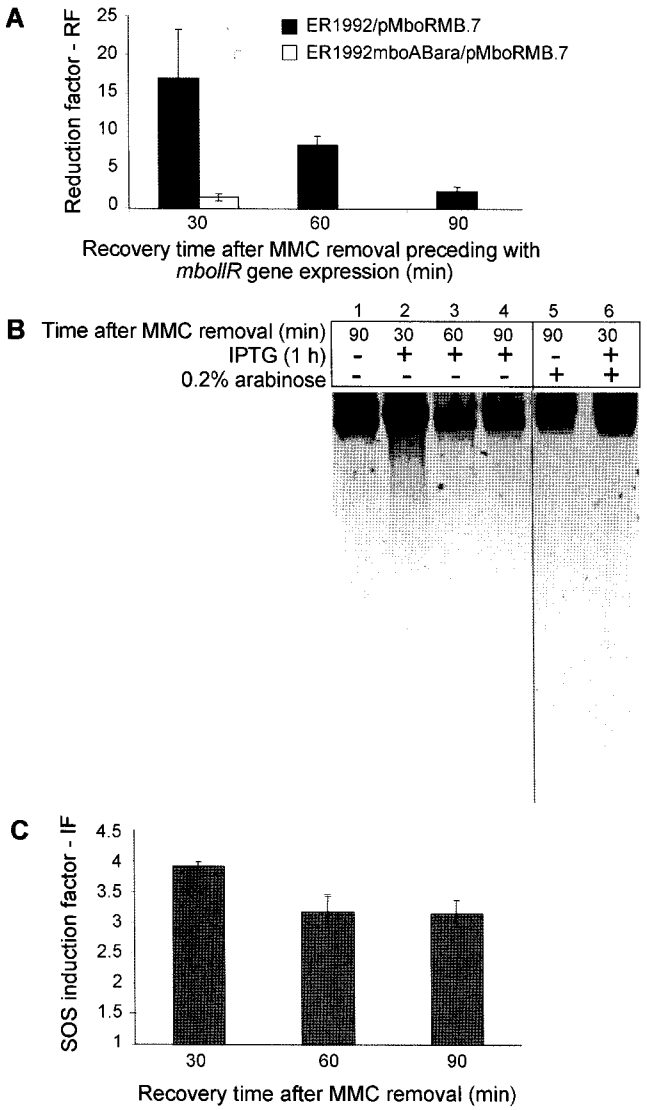

Fig. 2. Time-dependent sensitivity of SOS-repaired DNA to restriction. **A.** Relationship between duration of cell recovery after MMC removal and susceptibility of genomic DNA to autorestriction. Exponentially growing cells (37°C) were treated with MMC for 1 h, and upon MMC removal were allowed to grow for 30, 60, or 90 min, prior to 1 h IPTG (0.1 mM) induction to express the MboII $r^+m.2^+$ system. RF values were determined for each culture at the end of the IPTG induction time. The black color refers to *E. coli* ER1992/pMboRMB.7, and white refers to ER1992mboBAra/pMboRM.7 grown in the presence of 0.2% L-arabinose (induction of MboII MTases gene expression). Shown are the mean values and standard deviations of three independent experiments. **B.** Dependence of the degree of R.MboII-mediated DNA fragmentation on the duration of recovery after MMC removal. Chromosomal DNA was isolated from the 3-ml cultures of ER1992/pMboRMB.7 (lanes 1–4) or ER1992mboBAara/pMboRMB.7 (lanes 5 and 6), grown as described above, and their aliquots (20 µl) were resolved on 0.65% agarose gel electrophoresis with 0.5× Tris-borate-EDTA buffer, run overnight, followed by staining with ethidium bromide. The color of the picture is inverted for better visualization of the smear of DNA fragments. **C.** SOS-chromotest: determination of the SOS induction factor (IF) for cultures during the DNA repair process. ER1992 bacteria carrying both pMboRMB.7 and pANTSphoA plasmids were treated with MMC (1 h, 1.5 µM), and allowed further growth for 30, 60, or 90 min without MMC. Then, the levels of activity for β-galactosidase and alkaline phosphatase were estimated for treated and untreated bacteria and used to determine IFs.

unprotected MboII sites within the newly repaired DNA regions was highest in such cells. Comparative analysis of whole cellular DNA isolated from cultures after expression of the *mboIIR* gene revealed a smear of DNA fragments, indicative of its degradation *in vivo* (Fig. 2B). The most extensive DNA degradation was observed in the cells after the shortest time of recovery/methylation (Fig. 2B, lane 2), and noticeably less in a culture after 90 min of recovery (lane 4), as expected from the survival results. Almost no degradation was seen in the control sample grown for 90 min without IPTG induction (no restriction, lane 1) and in the case of cells fully protected against MboII restriction after L-arabinose induction of the *mboIIM* methyltransferasen genes in the ER1992mboBAra host (lanes 5 and 6).

To ascertain whether the requirement for high cellular DNA repair activity is vital just after MMC removal, we employed the SOS-chromotest. This assay directly expresses the level of SOS-response and genotoxicity [41]. Indeed, recovery for 30, 60, and 90 min after MMC treatment, but before IPTG induction of the *mboIIR*, showed that the highest level of SOS response is observed in a culture after 30 min of recovery time (IF=3.9±0.07; Fig. 2C). After a longer recovery, the response declined and stabilized at the level of 3.2±0.25 and 3.1±0.22 for 60 and 90 min of recovery, respectively (Fig. 2C). According to Khil and Camerini-Otero [26], a 10-min exposure to MMC, at a similar concentration level to ours, required 70 min for a drop in the induction signal and regaining of the basal expression for the key *recA* gene. Clearly, under the conditions that we applied, much more time was necessary to complete the DNA repair process (data not shown). Our results are also consistent with a kinetic of expression of the UV-induced *E. coli dinD* gene [12].

Bacteria Lacking an R-M System Tolerate Genotoxic Agent Conditions

We evaluated the genotoxic effect of MMC on *E. coli* strains containing or lacking R-M systems, cultivated under long-term MMC exposure. Assuming a common co-evolutionary history of constitutively expressed EcoRI R-M gene complexes and an *E. coli* host [51], compared with an unrelated MboII $r^+m.2^+$ inducible system, the former was employed for this purpose. Logarithmic-phase cultures of ER1992 cells and their derivatives carrying a chromosomally or plasmid-coded wild-type EcoRI R-M system (pANTSeco), respectively, were used. Bacteria were grown in LB medium at 37°C for 16 h, in the presence or absence of a sublethal concentration of MMC (0.75 µM), and c.f.u./ml was periodically determined for all cultures (Fig. 3). The survival of the R-M-carrying bacteria was significantly lower compared with those that lacked an R-M system. The relative viability of the ER1992pE and ER1992E bacteria was 3.2- and 8-times lower than those in the case of ER1992 (0.13±0.08 and 0.05±0.02 vs. 0.42±0.14, respectively).

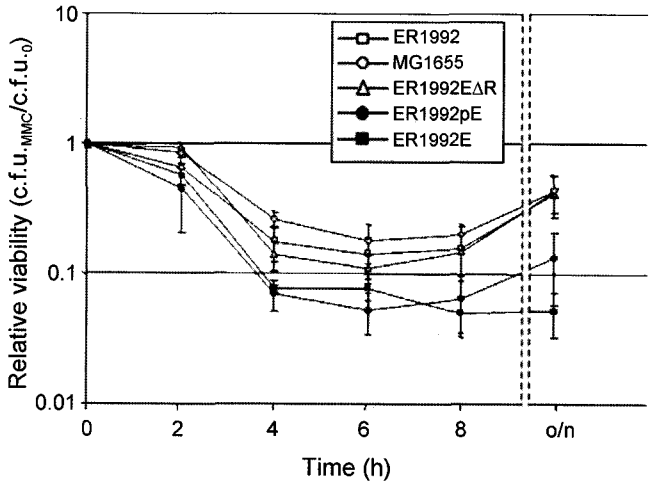

Fig. 3. Comparison of viability of isogenic strains with and without a type II R-M system during MMC exposure.
E. coli strains ER1992 (r^-m^-, open square), ER1992E (EcoRI r^+m^+, filled square), ER1992EΔR (EcoRI r^-m^+, open triangle), MG1655 (EcoKI r^+m^+, open circle), and ER1992pE (pANTSeco - EcoRI r^+m^+, filled circle) were grown overnight at 37°C with or without MMC (0.75 μM). The relative viability of each strain under MMC exposure was measured as a ratio of c.f.u./ml, determined after the addition of MMC agent ($c.f.u._{MMC}$), versus the c.f.u./ml of culture without MMC ($c.f.u._0$) at each measured point. All values are means±SD for at least three experiments. O/n: overnight.

The results obtained for ER1992EΔR bacteria possessing an R.EcoRI-deficient EcoRI R-M system, used as a negative control, clearly show that they behaved in a similar fashion to the ones lacking R-M (Fig. 3). Our findings suggested that the presence of REase activity significantly affected cell viability under MMC exposure growth conditions. This indicates that type II REase-proficient *E. coli* strains are more sensitive to genotoxic stress due to the substrates for resident restriction activity generated by the SOS-induced DNA repair process. Surprisingly, the drop in cell survival in the case of the ER1992pE (plasmid location of the R-M system) was much lower than expected (3.2 times). This might be a result of a higher methyltransferase concentration/activity, which creates a safe functional balance, rather than the loss of the plasmid (*i.e.*, Tet^S phenotype). We found that the pANTSeco plasmid was very stably maintained (data not shown). Moreover, all the lysates from the random Tet^R clones that we checked appeared to be EcoRI-restriction proficient and specifically cleaved the testing plasmid *in vitro* (data not shown). Interestingly, we learnt far more from this fact; namely, that cells maintaining the plasmid-expressed EcoRI R-M system (high level of activity) are more tolerant of occasional fluctuations in the balance between modification and restriction than in the case of its chromosomal expression (low level of activity). In marked contrast, a noncontrollable activity of type I EcoKI REase is alleviated under a UV-inducible mechanism [27, 48], which protects the chromosome when recombination generates DNA products with unmodified target sequences [3]. This takes place by means of post-translational ClpXP-dependent degradation of the HsdR subunit [31]. Indeed, analysis of the survival profile of MG1655 (EcoKI r^+m^+) and ER1992 (ΔEcoKI) cells, showed that they were equally tolerant of MMC exposure (Fig. 3).

Our findings again give rise to the question of the overall impact of R-M systems on genome organization and host cell fitness under different growth conditions (for a comprehensive discussion see [28]). Apparently, a resident R-M system is beneficial for the host as a defense against invading DNA and is also a prerequisite for some types of recombination processes. Inversely, REase as a toxic part of the system can be deleterious to its host under certain conditions. Therefore, the post-disturbance killing activity found in many R-M systems must, after all, force selective pressure in their hosts, which ensures the inheritance of these selfish genetic elements, and as a by-product, creates a broad spectrum of variability in the virulence of R-M systems [28]. Thinking about R-M systems as mobilizable genetic elements, we hypothesize that the perturbance in the functional balance of given R-M genes can happen at distinct moments anytime during the history of the R-M system "host visit", beginning from the self-establishment event, throughout the host DNA replication cycle(s), up to the loss of the residing gene complex. We show here that chromosome breakage made by an endonuclease as a reaction to the sudden appearance of a large number of unprotected recognition sites might have an adverse influence on cell viability in the short term. We postulate that this phenomenon would play a part in the process of the shaping of a stable co-adaptation between the host and its addiction system in the long term. This is applicable especially when the host and R-M genes represent a relatively recent relationship and are distantly related in terms of biochemical characteristics and genetic context (*e.g.*, a discrepancy in the consensus sequence of R-M promoters or inadequate codon usage).

In the experiment with the MMC-exposed bacteria, we focused upon the interference of the DNA repair process with modification and restriction. We found that autorestriction decreases the viability of bacterial cells under MMC-induced DNA repair conditions. We showed that the highest restriction-mediated lethal effect was observed when newly repaired, nonmethylated regions of DNA appeared. This was the result of the cellular response to MMC-triggered DNA damage, monitored by the SOS-chromotest.

Acknowledgments

We would like to thank New England Biolabs for sharing the *E. coli* ER1992 strain. The authors are grateful to Dr. B. Müller-Hill for the gift of the pMboR3.0 and pMboM1.1 plasmids. We would like to thank Martin

Blaszk for his excellent help with editing this manuscript. This work was supported by grants N301 066 31/1985 and BW/1470-5-0389-8 from the Ministry of Science and Higher Education (Warsaw, Poland).

REFERENCES

1. Aras, R. A., A. J. Small, T. Ando, and M. J. Blaser. 2002. *Helicobacter pylori* interstrain restriction–modification diversity prevents genome subversion by chromosomal DNA from competing strain. *Nucleic Acids Res.* **30:** 5391–5397.
2. Aras, R. A., T. Takata, T. Ando, A. var der Ende, and M. J. Blaser. 2001. Regulation of the *Hpy*II restriction–modification system of *Helicobacter pylori* by gene deletion and horizontal reconstitution. *Mol. Microbiol.* **42:** 369–382.
3. Blakely, G. W. and N. E. Murray. 2006. Control of the endonuclease activity of type I restriction–modification systems is required to maintain chromosome integrity following homologous recombination. *Mol. Microbiol.* **60:** 883–893.
4. Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, *et al.* 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277:** 1453–1462.
5. Bocklage, H., K. Heeger, and B. Muller-Hill. 1991. Cloning and characterization of the MboII restriction–modification system. *Nucleic Acids Res.* **19:** 1007–1013.
6. Bolivar, F., R. L. Rodriguez, M. C. Betlach, and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2:** 95–113.
7. Boyer, H. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* **41:** 459–472.
8. Cerritelli, S., S. S. Springhorn, and S. A. Lacks. 1989. DpnA, a methylase for single-strand DNA in the DpnII restriction system and its biological function. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 9223–9227.
9. Chang, A. C. Y. and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmid. *J. Bacteriol.* **134:** 1141–1156.
10. Cheng, S. C., R. Kim, K. King, S. H. Kim, and P. Modrich. 1984. Isolation of gram quantities of EcoRI restriction and modification enzymes from an overproducing strain. *J. Biol. Chem.* **259:** 11571–11575.
11. Cohen, S. N., A. C. Y. Chang, H. W. Boyer, and R. B. Helling. 1973. Construction of biologically functional bacterial plasmids *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **70:** 3240–3244.
12. Courcelle, J., A. Khodursky, B. Peter, P. O. Brown, and P. C. Hanawalt. 2001. Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*. *Genetics* **158:** 41–64.
13. Cromie, G. A. and D. R. F. Leach. 2001. Recombinational repair of chromosomal DNA double-strand breaks generated by a restriction endonuclease. *Mol. Microbiol.* **4:** 873–883.
14. Dronkert, M. L. G. and R. Kanaar. 2001. Repair of DNA interstrand cross-links. *Mutat. Res.* **486:** 217–247.
15. Fomenkov, A., J. P. Xiao, D. Dila, E. Raleigh, and S. Y. Xu. 1994. The "endo-blue method" for direct cloning of restriction endonuclease genes in *E. coli*. *Nucleic Acids Res.* **22:** 2399–2403.
16. Furmanek-Blaszk, B., R. Boratynski, N. Zolcinska, and M. Sektas. 2009. M1.MboII and M2.MboII type IIS methyltransferases: Different specificities, the same target. *Microbiology* **155:** 1111–1121.
17. Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177:** 4121–4130.
18. Handa, N., A. Ichige, and I. Kobayashi. 2009. Contribution of RecFOR machinery of homologous recombination to cell survival after loss of a restriction-modification gene complex. *Microbiology* **155:** 2320–2332.
19. Handa, N., A. Ichige, K. Kusano, and I. Kobayashi. 2000. Cellular responses to postsegregational killing by restriction–modification genes. *J. Bacteriol.* **182:** 2218–2229.
20. Handa, N. and I. Kobayashi. 1999. Post-segregational killing by restriction modification gene complexes: Observations of individual cell deaths. *Biochimie* **81:** 931–938.
21. Hasan, N., M. Koob, and W. Szybalski. 1994. *Escherichia coli* genome targeting. I. Cre-lox-mediated *in vitro* generation of ori$^-$ plasmids and their *in vivo* chromosomal integration and retrieval. *Gene* **150:** 51–56.
22. Heitman, J., T. Ivanenko, and A. Kiss. 1999. DNA nicks inflicted by restriction endonucleases are repaired by a RecA- and RecB-dependent pathway in *Escherichia coli*. *Mol. Microbiol.* **33:** 1141–1151.
23. Heitman, J., N. D. Zinder, and P. Model. 1989. Repair of the *Escherichia coli* chromosome after *in vivo* scission by the EcoRI endonuclease. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 2281–2285.
24. Ichige, A. and I. Kobayashi. 2005. Stability of EcoRI restriction–modification enzymes *in vivo* differentiates the EcoRI restriction–modification system from other postsegregational cell killing system. *J. Bacteriol.* **187:** 6612–6621.
25. Kaczorowski, T., M. Sektas, P. Skowron, and A. Podhajska. 1999. The FokI methyltransferase from *Flavobacterium okeanokoites*: Purification and characterization of the enzyme and its truncated derivatives. *Mol. Biotechnol.* **13:** 1–15.
26. Khil, P. P. and R. D. Camerini-Otero. 2002. Over 1000 genes are involved in the DNA damage response of *Escherichia coli*. *Mol. Microbiol.* **44:** 89–105.
27. Kelleher, J. E. and E. A. Raleigh. 1994. Response to UV damage by four *Escherichia coli* K-12 restriction systems. *J. Bacteriol.* **176:** 5888–5896.
28. Kobayashi, I. 2004. Restriction–modification systems as minimal forms of life, pp. 19–62. *In* A. Pingoud (ed.). *Nucleic Acids and Molecular Biology*, Vol. 14. *Restriction Endonucleases*. Springer-Verlag, Berlin Heidelberg.
29. Kumar, S., R. Lipman, and M. Tomasz. 1992. Recognition of specific DNA sequences by mitomycin C for alkylation. *Biochemistry* **31:** 1399–1407.
30. Lin, L. F., J. Posfai, R. J. Roberts, and H. Kong. 2001. Comparative genomics of the restriction–modification systems in *Helicobacter pylori*. *Proc. Natl. Acad. Sci. U.S.A.* **98:** 2740–2745.
31. Makovets, S., V. A. Doronina, and N. E. Murray. 1999. Regulation of endonuclease activity by proteolysis prevents breakage of

unmodified bacterial chromosomes by type I restriction enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **96:** 9757–9762.
32. McClelland, M., M. Nelson, and C. R. Cantor. 1985. Purification of MboII methylase (GAAGmA) from *Moraxella bovis*: Site specific cleavage of DNA at nine and ten base pair sequences. *Nucleic Acids Res.* **13:** 7171–7182.
33. Merkiene, E., G. Vilkaitis, and S Klimasauskas. 1998. A pair of single-strand and double-strand DNA cytosine-N4 methyltransferases from *Bacillus centrosporus*. *Biol. Chem.* **379:** 569–571.
34. Miller, J. H. 1972. *Experiments in Molecular Genetics*, p. 439. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. Mruk, I. and R. M. Blumenthal. 2008. Real-time kinetics of restriction–modification gene expression after entry into a new host cell. *Nucleic Acids Res.* **36:** 2581–2593.
36. Nagornykh, M. O., E. S. Bogdanova, A. S. Protsenko, A. S. Solonin, M. V. Zakharova, and K. V. Severinov. 2008. Regulation of gene expression in a type II restriction–modification system. *Russ. J. Genetics* **44:** 523–532.
37. Naito, Y., K. Kusano, and I. Kobayashi, I. 1995. Selfish behavior of restriction–modification systems. *Science* **267:** 897–899.
38. Nakayama, Y. and I. Kobayashi. 1998. Restriction–modification gene complexes as selfish gene entities: Roles of a regulatory system in their establishment, maintenance, and apoptotic mutual exclusion. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 6442–6447.
39. Nobusato, A., I. Uchiyama, S. Ohashi, and I. Kobayashi. 2000. Insertion with target duplication: A mechanism for gene mobility suggested from comparison of two related bacterial genomes. *Gene* **259:** 99–108.
40. Posfai, G., M. Koob, Z. Hradecna, N. Hasan, M. Filutowicz, and W. Szybalski. 1994. *In vivo* excision and amplification of large segments of the *Escherichia coli* genome. *Nucleic Acids Res.* **22:** 2392–2398.
41. Quillardet, P., O. Huisman, R. D'Ari, and M. Hofnung. 1982. SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* **79:** 5971–5975.
42. Roberts, R. J., T. Vincze, J. Posfai, and D. Macelis. 2007. REBASE: Restriction enzymes and DNA methyltransferases. *Nucleic Acids Res.* **33:** D230–D232.
43. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
44. Spira, B. and T. Ferenci. 2008. Alkaline phosphatase as a reporter of sigma(S) levels and *rpoS* polymorphisms in different *E. coli* strains. *Arch. Microbiol.* **189:** 43–47.
45. Surby, M. A. and N. O. Reich. 1996. Contribution of facilitated diffusion and processive catalysis to enzyme efficiency: Implications for the EcoRI restriction–modification system. *Biochemistry* **35:** 2201–2208.
46. Surby, M. A. and N. O. Reich. 1996. Facilitated diffusion of the EcoRI DNA methyltransferase is described by a novel mechanism. *Biochemistry* **35:** 2209–2217.
47. Taylor, J. D., A. J. Goodall, C. L. Vermote, and S. E. Halford. 1990. Fidelity of DNA recognition by the EcoRV restriction/modification system *in vivo*. *Biochemistry* **29:** 10727–10733.
48. Thoms, B. and W. Wackernagel. 1982. UV-induced alleviation of λ restriction in *Escherichia coli* K12: Kinetics of induction and specificity of this SOS function. *Mol. Gen. Genet.* **186:** 111–117.
49. Tock, M. R. and D. T. F. Dryden. 2005. The biology of restriction and anti-restriction. *Curr. Opin. Microbiol.* **8:** 466–472.
50. Yanisch-Perron, C., J Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119.
51. Yoshimori, R., D. Roulland-Dussoix, and H. W. Boyer. 1972. R-Factor-controlled restriction and modification of deoxyribonucleic acid: Restriction mutants. *J. Bacteriol.* **112:** 1275–1279.