Cellular localization and dynamics of the Mrr type IV restriction endonuclease of Escherichia coli

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ABSTRACT
In this study, we examined the intracellular whereabouts of Mrr, a cryptic type IV restriction endonuclease of Escherichia coli K12, in response to different conditions. In absence of stimuli triggering its activity, Mrr was found to be strongly associated with the nucleoid as a number of discrete foci, suggesting the presence of Mrr hotspots on the chromosome. Previously established elicitors of Mrr activity, such as exposure to high hydrostatic pressure (HP) or expression of the Hhal methyltransferase, both caused nucleoid condensation and an unexpected coalescence of Mrr foci. However, although the resulting Mrr/nucleoid complex was stable when triggered with Hhal, it tended to be only short-lived when elicited with HP. Moreover, HP-mediated activation of Mrr typically led to cellular blebbing, suggesting a link between chromosome and cellular integrity. Interestingly, Mrr variants could be isolated that were specifically compromised in either HhaII- or HP-dependent activation, underscoring a mechanistic difference in the way both triggers activate Mrr. In general, our results reveal that Mrr can take part in complex spatial distributions on the nucleoid and can be engaged in distinct modes of activity.

INTRODUCTION
Bacterial type IV restriction endonucleases (REases), unlike those belonging to other types, have no cognate methyltransferase (MTase) and bear specificity for modified DNA in certain sequence contexts (1,2). The cellular function of these solitary enzymes is often cryptic, although some of them have been shown to affect the entry of foreign DNA such as phages (3,4). In this context, their ability to target modified DNA is seen as a strategy to deal with the increasingly complex chromosome decorations that have evolved in some phages to escape restriction (5). Another hypothesis, however, suggests type IV REases to ward off the establishment of foreign MTases that might impose an altered epigenetic regulation on the host (6,7).

Escherichia coli K12 encodes for a number of type IV REases (McrA, McrBC and Mrr), all of which originate from laterally acquired genetic elements such as the e14 element (encoding mcrA) and the ‘immigration control region’ (encoding mcrBC and mrr) (8–12). While McrA and McrBC have previously been shown to be involved in restricting phage infection (3,4), Mrr was originally discovered as a peculiar enzyme conferring genotoxicity on heterologous expression of some exotic type II MTases in E. coli K12 (9,13). During the last years, however, more insights into the cellular impact and biological significance of Mrr have been revealed. In this context, a first important finding entailed the identification of Mrr as dedicated trigger of the high (hydrostatic) pressure (HP)-induced SOS response and its concomitant phenotypes in E. coli K12 (14–18). Mechanistically, it was inferred that Mrr generated double-strand breaks in the host chromosome specifically in the presence of sublethal HP stress (~100 MPa) (15), although it currently remains unclear how the physical perception of HP by the cell eventually elicits Mrr activity. Although a number of restriction alleviation mechanisms have been discovered that prevent self-digestion of the chromosome in adverse conditions (19,20), HP activation of Mrr presents the first case in which host DNA integrity is deliberately affected in response to stress (21).

A second finding concerned the recent observation that Mrr activity of E. coli K12 could be triggered by type III MTases (i.e. Mod proteins) of close relatives such as E. coli ED1A and Salmonella Typhimurium LT2 (22), possibly forwarding these MTases as natural elicitors of Mrr activity. Moreover, acquisition of Mrr could readily drive the loss of endogenous Mod activity in these strains, and subsequent bioinformatics analysis suggested that the mutual antagonism between homologs of Mrr and Mod could even extend beyond these species (22).
To better understand the behavior and impact of this peculiar endonuclease, this report focuses on the whereabouts and dynamics of the Mrr REase inside the cell, and reveals that it is strongly associated with the nucleoid, with its localization differing depending on the conditions eliciting its activity.

MATERIALS AND METHODS

Strains and growth conditions

*E. coli* K12 MG1655 (23) was used as a parental strain in this study. Its *mrr::Kn* and *mrr::Tc* derivatives were obtained through *in vitro* (EZ-Tn5 transposon kit; Epicentre, Landgraaf, The Netherlands) (22) or *in vivo* (24) transposon mutagenesis, respectively, whereas its *lon::Kn* derivative was constructed as described in (17). Strain MG1655 *mrr::Tc iseR::yfp* was constructed by pKD46-based recombineering (25) a polymerase chain reaction (PCR) ampiclon of the *iseR::yfp-frt-cat-frt* fragment (obtained using primers 5'-ATGTCAGACTTG-3' and 5'-CTCCGATCATCACTCAAT GC-3' on *S. Typhimurium* LT2 *iseR::yfp-frt-cat-frt* genomic DNA; Passaris et al., in preparation) into MG1655 *mrr::Tc*, and subsequently flipping out the *cat* marker gene using pCP20 (26). Strains were transformed with the appropriate plasmids (see later in the text) by electroporation, whereas curing of temperature-sensitive plasmids [i.e. pKD46 and pCP20; (25,26)] was performed by growing the corresponding strain at the non-permissive temperature in the absence of plasmid selection, and subsequently isolating a clone that had lost the plasmid.

Stationary phase *E. coli* cultures were obtained by growth in lysogeny broth (LB) (27) for 21 h at 37°C under well-aerated conditions. Late exponential phase cultures were obtained by diluting stationary phase cultures 1/1000 in fresh prewarmed LB and allowing further incubation until late exponential phase (OD600 = 0.6) as described earlier (14). When necessary, the following chemicals (Applichem, Darmstadt, Germany) were added to the growth medium to obtain the indicated final concentrations: 100 μg/ml ampicillin (Ap100), 30 μg/ml chloramphenicol (Cm30), 50 μg/ml kanamycin (Kn50), 20 μg/ml tetracycline (Tc25), 1 μg/ml mitomycin C, 1 mM IPTG (isopropyl β-D-1-thiogalacto-pyranoside), 0.02% arabinose and/or 0.1% glucose.

Screening for Mrr variants with altered activity

A first screen was designed to obtain a mutant Mrr protein that would not be activated by HP shock, but would still react to the methylation imprint imposed by *Mod*LT2 or HhaII. For this, 40 independent lineages of MG1655 *mrr::Kn* equipped with pAA810 [pACYC184 backbone expressing wild-type *mrr* from its native promotor; (15)] were iteratively exposed to HP shock with intermediate resuscitation and outgrowth of the survivors. After 3–4 of such cycles, the severe lethality stemming from HP-induced Mrr activity selected for the enrichment of clones carrying compromised *mrr* alleles that would not respond to HP exposure anymore. The plasmids of such clones were isolated and transformed to *S. Typhimurium* LT2 K2 [an SOS indicator strain of LT2 described previously (28)], to examine which of the plasmid-borne *mrr* alleles still retained the functionality to react to the methylation imprint imposed by the endogenous *Mod*LT2 activity in LT2. While most *mrr* alleles proved to be compromised in both activities, one allele (i.e. *mrrT518C*) could be found in which a T518C mutation (resulting in a VI73A substitution in the Mrr protein) abolished HP activation of the resulting MrrV173A variant, while preserving its reactivity to *Mod*LT2 and HhaII activity.

Next, a second screen was designed to obtain a mutant Mrr protein that would not be activated by the methylation imprint imposed by *Mod*LT2 or HhaII but would still react to HP shock. For this screen, 20 independent lineages of *S. Typhimurium* LT2 equipped with pAA810 were grown for a number of generations, until the absence of cell lysis (due to endogenous prophage activation) indicated the alleviation of the Mrr/ModLT2 antagonism (22). From this screen, one *mrr* allele (i.e. *mrrC835T*) could be found in which a C835T mutation (resulting in a H279Y substitution in the Mrr protein) compromised HhaII-mediated activation of the resulting MrrH279Y variant, while preserving its reactivity to HP shock.

Construction of plasmids

Plasmid pBAD-gfp::mrr encodes the Gfp-Mrr fusion protein (with the Gfp moiety located N-terminally of Mrr) under control of the arabinose-inducible PBAD promoter and was constructed by digesting a PCR ampiclon of *mrr* (obtained using primers 5'-ATGTCAGACTTG-3' and 5'-CGATAAGCTTGCCTGCGGGGTAG-3' on *E. coli* MG1655 genomic DNA) with PstI and HindIII, before ligation in the low copy number pBAD33-gfp mut2-T7tag plasmid (29). Plasmids pBAD-gfp::mrrT518C and pBAD-gfp::mrrC835T were constructed in the same fashion and encode Gfp-MrrV173A and Gfp-MrrH279Y fusion proteins, respectively.

Control plasmid pBAD-gfp-mrr encodes Gfp and Mrr individually (i.e. unfused to each other) under control of the arabinose-inducible PBAD promoter and was constructed by digesting a PCR ampiclon of *mrr* (obtained using primers 5'-ACTGCTGACGTATAAACGCTGCA AGGATCTACTGACGGG3'-3' and 5'-CGATAAGCTTGCCTGCGGGGTAG-3' on *E. coli* MG1655 genomic DNA) with PstI and HindIII, before ligation in pBAD33-gfp mut2-T7tag digested with the same enzymes.

Plasmid pTRC-modED1a encodes the *E. coli* ED1A type III *Mod* MTase [i.e. *Mod*ED1A; (30)] under control of the IPTG-inducible P*rec* promoter and was constructed by digesting a PCR ampiclon of *mrr* (obtained using primers 5'-ACTGCTGACGTATAAACGCTGCA AGGATCTACTGACGGG3'-3' and 5'-CGATAAGCTTGCCTGCGGGGTAG-3' on *E. coli* MG1655 genomic DNA) with XbaI and PstI, before ligation in pTRC99A (31) digested with the same enzymes.

Plasmid pTRC-modLT2 encodes the *S. Typhimurium* LT2 type III (*StyLTI*) *Mod* MTase [i.e. *Mod*LT2; (32)] under control of the IPTG-inducible P*rec* promoter and was constructed by digesting a PCR ampiclon of
the mod\textsuperscript{L2} gene (obtained using primers 5'-AAGTTCTAGAGGACGGAAGATACATGTTG-3' and 5'-CGTAAAGCTTTATCATCCTTAAATACG-3' on S. Typhimurium LT2 genomic DNA) with XbaI and HindIII, before ligation in pTRC99A digested with the same enzymes.

Plasmid pTRC-hhaII encodes the Haemophilus haemolyticus HhaII MTase under control of the IPTG-inducible P\textsubscript{BAD} promoter and was constructed by digesting a PCR amplicon of hhaII (obtained using primers 5'-ACGCTCTAGAGCAATATTTAGTTCTTTCC-3' and 5'-CGTACTGCAGCGTAACGCATTACTAATC-3' on pSK5 (33)) with XbaI and PstI, before ligation in pTRC99A. The hhaII gene in pTRC-hhaII incurred a single T645C base transition compared with the corresponding GenBank sequence, although this mutation is silent and does not affect the amino acid sequence of HhaII. Furthermore, IPTG induction of pTRC-hhaII in E. coli MG1655 protected the plasmid and chromosome from subsequent HinfI endonuclease activity \textit{in vitro} (data not shown), further supporting wild-type functionality of the expressed HhaII protein.

Plasmid pACYC184-P\textsubscript{BAD}-mrr was constructed earlier (22) and consists of a pACYC184 backbone equipped with the MG1655 mrr gene under control of the P\textsubscript{BAD} promoter.

Plasmids were transformed to the appropriate bacterial strains via electroporation, and induction of the P\textsubscript{BAD} or P\textsubscript{BAD} endo promoter in the resulting strains was instigated with 0.02% arabinose or 1 mM IPTG, respectively, 1 h before harvesting the corresponding late exponential phase cultures.

High hydrostatic pressure treatment
HP treatment was essentially performed as described previously (14). Briefly, cells from a late exponential phase culture were harvested by centrifugation (6000 \(\times\)g, 5 min) and resuspended in an equal volume of fresh prewarmed LB. Subsequently, a 100 \(\mu\)l portion of resuspended cells was heat sealed in a sterile polyethylene bag after exclusion of the air bubbles and subjected to 100 MPa for 10–15 min in an 8-ml pressure vessel (HP1U-10000, 95/1994; Resato, Roden, The Netherlands), held at 20°C with an external water jacket connected to a cryostat. After HHP exposure, the culture was aseptically retrieved from the polyethylene bag and used for viability determination and/or microscopy.

Determination of viability
Serial dilutions of culture samples were plated or spotted on LB agar and incubated overnight at 37°C. Subsequently, colonies on the plates were counted and viability was expressed as Log \(N\), with \(N\) being the colony forming units (CFU) per ml of the sample.

Fluorescence microscopy
Fluorescence microscopy was performed with a temperature-controlled (Okolab Ottaviano, Italy) Ti-Eclipse inverted microscope (Nikon, Champigny-sur-Marne, France) equipped with a TI-CT-E motorized condenser, a Gfp filter (Ex 472/30 nm DM 495 Em 520/35), a 4',6-diamidino-2-phenylindole (DAPI) filter (Ex 377/50 nm Dm 409 Em 447/60), a Yfp filter (Ex 500/24, DM 520, Em 542/27) and a CoolSnap HQ2 FireWire CCD-camera. For imaging, cells were placed between LB agar pads and a cover glass, essentially as described previously (34), and incubated at 37°C. Representative images were acquired using NIS-Elements (Nikon) and further handled with open source software ImageJ (downloaded from http://rsbweb.nih.gov/ij/).

Sequencing
Sequencings to validate genetic constructs were performed by Macrogen Europe (Amsterdam, The Netherlands).

RESULTS

Mrr is localized as distinct foci on the nucleoid
To establish its whereabouts in the cell, the open reading frame of the E. coli K12 MG1655 (further referred to as MG1655) mrr gene was translationally fused to the 3’ end of the gene encoding the green fluorescent protein (gfp), and this construct was conditionally expressed in MG1655 mrr::Kn from a low copy number plasmid (pBAD-gfp::mrr) via the arabinose-inducible P\textsubscript{BAD} promoter (with 0.02% arabinose). The resulting Gfp-Mrr fusion protein behaved phenotypically similar to the wild-type Mrr protein (i.e. not fused to Gfp), and on expression only conferred genotoxicity in the presence of specific MTase activity or after HP (100 MPa) shock (see later in the text).

Having validated the wild-type functionality of the Gfp-Mrr fusion protein, its location inside the cell was examined with fluorescence microscopy. Interestingly, this revealed the Gfp-Mrr protein to be organized as distinct foci on the nucleoid (Figure 1A–C). To further corroborate this association, Gfp-Mrr was expressed in a lon mutant of MG1655, a genetic background that tends to filament excessively in the presence of mitomycin C (35). In such filaments, where the DAPI-stained nucleoid is more clearly distinguishable from the surrounding cytoplasm, Gfp-Mrr was observed to colocalize almost strictly with the nucleoid (Figure 1D–E).

Effect of HP on Mrr localization
Since the perception of mild HP stress (100 MPa) has previously been identified as a strong elicitor of Mrr activity (15), its impact on Gfp-Mrr localization was examined. Remarkably, within 15–30 min after HP exposure, Gfp-Mrr activity caused nucleoid condensation, with the endonuclease coalescing in the mid of the condensed nucleoid (Figure 2A and B). Moreover, longer cells that were able to divide often revealed two of such centralized Gfp-Mrr foci moving toward each other and coalescing in the mid of the cell (Figure 2C), indicating a retrograde transport of their segregating nucleoids after condensation. In addition, HP-induced Gfp-Mrr centralization was often transient, with the central Gfp-Mrr focus gradually disintegrating and dispersing in the cytoplasm within 30–
60 min post-HP shock, perhaps indicative for the loss of nucleoid integrity (Figure 2D). As a result of this HP/Mrr activation, cellular viability also dropped >100-fold (Supplementary Figure S1).

To further interpret HP-induced Gfp-Mrr dynamics, the corresponding nucleoid dynamics were monitored using an endogenous fluorescent nucleoid reporter (i.e. IscR-Yfp) recently constructed and validated in our group (Passaris et al., in preparation), rather than with UV-excited DAPI staining that might interfere with the proper dynamics during time-lapse exposures. Upon HP/Mrr activation, this reporter likewise revealed (i) condensation and subsequent dispersal of the nucleoid (Figure 3A) as well as (ii) retrograde transport of segregated nucleoids in dividing cells (Figure 3B) to take place, thereby corroborating the observations made with Gfp-Mrr. The persistent nucleoid-wide distribution of the IscR-Yfp fusion protein throughout these experiments indicates that nucleoid condensation does not force nucleoid-associated proteins in general to coalesce mid-nucleoid by default.

Finally, it was surprising that starting at ~45–60 min after HP shock, many of the cells suffered a sort of cytoplasmic blebbing that compromised further growth and Mrr/nucleoid dynamics (Figures 2D and 3A, white arrows), although the underlying mechanisms behind this phenotype so far remain unclear.

Neither nucleoid condensation nor blebbing were visible after HP shock in the absence of Mrr expression (after which cells resumed regular growth, nucleoid segregation and division; data not shown), indicating these phenotypes stem from HP/Mrr-mediated genotoxicity.

**Effect of Mod and HhaII on Mrr localization**

As a modification-dependent type IV REase, Mrr has previously also been shown to react to the activity of MTases such as the type III (Mod) enzymes from *E. coli* ED1A (ModED1A) and *S. Typhimurium* LT2 (ModLT2) (22), and the type II HhaII enzyme from *H. haemolyticus* (9). On investigation of the impact of these MTases on Mrr localization, we found that both ModED1A and ModLT2 activity in part displaced Gfp-Mrr from its characteristic foci (Figure 4A) toward a more cloud-like distribution on the nucleoid (Figure 4B and C), likely reflecting the appearance of new binding sites on the chromosome. However, cellular viability was not affected by eliciting Mrr activity in this fashion (Supplementary Figure S2).

In contrast, in the presence of HhaII MTase activity, Gfp-Mrr caused strong nucleoid condensation and assumed a single focal position on the nucleoid (Figure 4D), similar to its reaction to HP shock described earlier in the text (Figure 2B). Moreover, cellular viability also dropped >10-fold as a result of HhaII/Mrr activation (Supplementary Figure S2). In further comparison to HP/
Mrr activation, however, HhaII/Mrr activation caused a more stable association between Mrr and the condensed nucleoid. In fact, longer coexpression of HhaII and Gfp-Mrr did not resolve this constellation, indicating that the nucleoid does not become fully degraded or dispersed (data not shown). In addition, loss of cellular integrity after HhaII/Mrr activation could be observed as well (data not shown), although to a lesser extent and in a more heterogeneous fashion compared with the cellular blebbing observed after HP/Mrr activation.

Nucleoid condensation did not occur upon expression of HhaII alone (data not shown), underscoring that the activity of this MTase itself is not genotoxic in the absence of Mrr.

**Figure 2.** (A, B) Localization of Gfp-Mrr and the nucleoid (stained with DAPI) in (A) unstressed or (B) HP-stressed MG1655 *mrr*::*Kn* pBAD-gfp::mrr cells (induced with 0.02% arabinose). Consecutive panels correspond to phase contrast, Gfp, DAPI and merged images of the same cells. (C) Intracellular movement of coalesced Gfp-Mrr foci in a nearly divided MG1655 *mrr*::*Kn* pBAD-gfp::mrr cell (induced with 0.02% arabinose) at indicated time points after HP exposure. Phase contrast and Gfp images were merged. (D) Coalescence and subsequent dispersion of Gfp-Mrr in MG1655 *mrr*::*Kn* pBAD-gfp::mrr cells (induced with 0.02% arabinose) at indicated time points after HP exposure. Phase contrast (upper panel) and Gfp (lower panel) images are shown. White arrows indicate when and where cells suffer from blebbing. The scale bar corresponds to 1 μm.
Mutational uncoupling of HhaII- and HP-induced activation of Mrr

Triggered by potential mechanistic differences between the activation of Gfp-Mrr by HP or HhaII, we examined whether one mode of activation could be compromised without affecting the other. More specifically, we devised a first screen looking for possible Mrr variants that would lose their ability to react to HP exposure, while nevertheless retaining their reactivity to HhaII exposure (‘Materials and Methods’ section). Interestingly, such a Mrr variant could be found (i.e. MrrV173A encoded by mrr_T518C) in which a single amino acid substitution abolished HP activation (Figure 5A and B, Supplementary Figure S1), but preserved HhaII activation (Figure 5C and D, Supplementary Figure S2), strongly indicating that HP activation of Mrr depends on structural features that are not required for its activation by HhaII-mediated methylation.

In a second screen, we conversely looked whether it was also possible to obtain Mrr variants that would lose their ability to react to HhaII methylation, without being affected in their reactivity to HP exposure (‘Materials and Methods’ section). From this screen, an Mrr variant (i.e. Mrr^H279Y encoded by mrr_C835T) could be isolated that, rather than causing chromosome condensation, assumed a cloud-like distribution over the nucleoid upon coexpression with HhaII (Figure 6C and D), perhaps suggesting that this variant still recognizes the target sites generated by HhaII activity without actually being able to cleave these sites and cause nucleoid condensation. Nevertheless, this deviant behavior still conferred some lethality (i.e. ~2-fold drop in cellular viability), although the latter was strongly attenuated compared with wild-type Mrr behavior (i.e. ~12-fold drop in cellular viability) (Supplementary Figure S2). Interestingly, however, this H279Y mutation did not prevent Gfp-Mrr to cause nucleoid condensation with concomitant central coalescence of the enzyme and massive lethality (>100-fold drop in viability) after HP shock (Figure 6A and B, Supplementary Figure S1).

DISCUSSION

Bacterial chromosomes associate with a great number of specific proteins and manifest themselves as densely and dynamically structured nucleoids inside the cell. While over the last years, the whereabouts and spatial orchestrations of nucleoid-associated proteins and processes involved in DNA replication, mismatch repair and chromosome segregation have been studied with (live) cell biology approaches (36–41), similar insights into the in vivo dynamics of restriction modification systems are still lacking.

In this study, we examined the intracellular localization and spatial dynamics of Mrr, a cryptic and laterally acquired type IV REase of E. coli K12 MG1655.

Figure 3. Nucleoid dynamics (A) in MG1655 mrr::Tc iscR-yfp pACYC184-P_{BAD}-mrr cells or (B) in a nearly divided MG1655 mrr::Tc iscR-yfp pACYC184-P_{BAD}-mrr cell at indicated time points after HP exposure. Phase contrast (upper panel) and Yfp (lower panel) images are shown. White arrows indicate when and where cells suffer from blebbing. The scale bar corresponds to 1 μm.
Figure 4. Localization of Gfp-Mrr and the nucleoid (stained with DAPI) in MG1655 mrr::Kn pBAD-gfp::mrr cells expressing (A) no foreign MTase (from empty pTRC99A), (B) the ModED1A MTase (from pTRC-modED1A), (C) the ModLT2 MTase (from pTRC-modLT2) or (D) the HhaII MTase (from pTRC-hhaII). Consecutive panels correspond to phase contrast, Gfp, DAPI and merged images of the same cells. The scale bar corresponds to 1 μm.
Figure 5. Localization of the Gfp-Mrr\textsuperscript{Y173A} variant and the nucleoid (stained with DAPI) in (A) unstressed or (B) HP-stressed MG1655 \textit{mrr::Kn} pBAD-gfp::\textit{mrr\textsuperscript{T518C}} cells, or in MG1655 \textit{mrr::Kn} pBAD-gfp::\textit{mrr\textsuperscript{T518C}} cells expressing (C) no foreign MTase (from empty pTRC99A) or (D) the HhaII MTase (from pTRC-hhaII). Consecutive panels correspond to phase contrast, Gfp, DAPI and merged images of the same cells. The scale bar corresponds to 1\,\mu m.
Interestingly, Mrr was not distributed throughout the cytoplasmic space, but was found to be strongly associated with the *E. coli* nucleoid. Moreover, under normal growth conditions (in the absence of any Mrr-dependent genotoxicity), it seemed to be organized as a number of distinct nucleoid-associated foci, which might indicate the presence of Mrr hotspots on the chromosome. Over the past few years, an increasing number of chromosome-related structures and processes have been shown to generate or occupy distinct topological domains on the nucleoid (39,41). RNA polymerase enzymes, for example, were shown to be gathered or tethered in distinct transcription complexes (42), whereas the nucleoid structuring H-NS protein is thought to play an important role in the formation of microdomain loops (38). As such, it is becoming increasingly clear that many of the nucleoid-associated proteins either generate or become attracted to nucleoid domains and superstructure, and our data indicate that Mrr (and perhaps other REases) might be susceptible to these forms of

![Figure 6. Localization of the Gfp-Mrr<sup>H279Y</sup> variant and the nucleoid (stained with DAPI) in (A) unstressed or (B) HP-stressed MG1655 *mrr::Kn* pBAD-gfp::<sup>C835T</sup>*mrr* cells, or in MG1655 *mrr::Kn* pBAD-gfp::<sup>C835T</sup>*mrr* cells expressing (C) no foreign MTase (from empty pTRC99A) or (D) the HhaII MTase (from pTRC-hhaII). Consecutive panels correspond to phase contrast, Gfp, DAPI and merged images of the same cells. The scale bar corresponds to 1 μm.
architectural organization as well. In fact, the mid-nucleoid localization of Mrr foci on HP/Mrr-inflicted chromosome condensation (whereas other nucleoid-associated proteins such as IscR-Yfp remain associated with the entire nucleoid) is in agreement with this hypothesis, as it seems to reflect an ordered gathering of Mrr-associated domains as opposed to a random wrapping process that would not enforce the coalescence of Mrr-associated regions during condensation of chromosomes. Although nucleoid condensation is a typical response to double-strand DNA breaks (43), in this case likely stemming from Mrr endonuclease activity, the underlying forces and mechanisms remain to be fully elucidated. Nevertheless, our data underscore the proficiency of this process, as it is able to retract nucleoids that are nearly completely segregated in dividing cells.

Interestingly, although nucleoid condensation and coalescence of Mrr occurs in response to HhaII/Mrr activation as well, the resulting nucleoids generally seem smaller compared with those observed after HP/Mrr activation, indicating that they are partly degraded and/or more densely packed. Furthermore, the HhaII/Mrr-induced nucleoid/Mrr complex appeared to be more stable than the complex induced by HP/Mrr activation, which tends to resolve rapidly into the cytoplasmic dispersion of the chromosome and Mrr. Importantly, mechanistic differences between both modes of activation are further underscored from a structural point of view as well, as certain amino acid substitutions in Mrr can specifically quench one mode of activation while leaving the other intact. As such, this mutational uncoupling of Mrr’s apparent dual reactivity implies that HP activation of the endonuclease in part depends on structural features that are irrelevant for its activation by the methylation pattern imposed by HhaII, and vice versa.

Interesting differences could also be observed between activation of Mrr through either Mod (i.e. ModED1A and ModLT2)-dependent or HhaII-dependent methylation. In sharp contrast to HhaII/Mrr (and HP/Mrr) activation, ModED1A/Mrr and ModLT2/Mrr activation both fail to impose nucleoid condensation (with Mrr coalescence) and cell death, but rather result in a modest displacement of Mrr from its characteristic distinct foci into a more cloud-like distribution on the nucleoid. As such, it could be hypothesized that perhaps the quality and/or number of target sites generated by ModED1A or ModLT2 is insufficient for Mrr to generate the amount of double-stranded breaks necessary to result in nucleoid condensation and concomitant cell death. In this context, it is important to note that (in contrast to the type II HhaII MTase) type III (Mod) MTases tend to cause only hemimethylation because of their typically asymmetric target site (44). Furthermore, compared with the displacement caused by ModED1A/Mrr and ModLT2/Mrr activation, the displacement of the MrrH279Y mutant in response to HhaII activity was much more pronounced, suggesting a larger number of (cleavable) Mrr target sites to be generated by HhaII activity. Since the (asymmetric) recognition sites of currently characterized type III MTases are typically 5′–6 bp (1,44) [with that of the Str/LTI MTase represented by ModLT2 being 5′-CAGAT-3′; (45)], the more generic and palindrome 5′-GANTC-3′ recognition site of the HhaII MTase (46) could lead to an order of magnitude more methylated sites, although it remains difficult to translate this into actual Mrr target sites as long as the recognition sequence of this REase remains obscure.

Finally, the observation that HP/Mrr-mediated genotoxicity specifically tends to trigger cellular blebbing seems to suggest a causal relation between DNA damage and loss of cellular integrity. In this context, a most recent study by Yitzhaki et al. (47) conversely demonstrated imposed envelope stress to result in DNA damage as well, further underscoring a functional connection between nucleoid and envelope integrity that warrants further investigation.

In general, our results reveal that REases can take part in complex spatial distributions on the nucleoid, and that these distributions can reflect mechanistic differences in mode of action.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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