3908–3918 Nucleic Acids Research, 2014, Vol. 42, No. 6 Published online 13 January 2014
doi:10.1093/nar/gkt1370

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

**Anirban Ghosh[1], Ioannis Passaris[1], Mehari Tesfazgi Mebrhatu[1], Susana Rocha[2], Kristof Vanoirbeek[1], Johan Hofkens[2] and Abram Aertsen[1,*]**

[1]Department of Microbial and Molecular Systems (M2S), Laboratory of Food Microbiology, KU Leuven, B-3001 Leuven, Belgium and [2]Department of Chemistry, KU Leuven, B-3001 Leuven, Belgium

Received October 28, 2013; Revised December 11, 2013; Accepted December 12, 2013

## 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 HhaII methyltransferase, both caused nucleoid condensation and an unexpected coalescence of Mrr foci. However, although the resulting Mrr/nucleoid complex was stable when triggered with HhaII, 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 whom correspondence should be addressed. Tel: +32 16 32 17 52; Fax: +32 16 32 19 60; Email: abram.aertsen@biw.kuleuven.be

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 transposome 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 iscR::yfp* was constructed by pKD46-based recombineering (25) a polymerase chain reaction (PCR) amplicon of the *iscR::yfp-frt-cat-frt* fragment (obtained using primers 5′-ATGTCAGACTTG TCCCTGCT-3′ and 5′-CTCCGTACATCACTCAAT GC-3′ on *S.* Typhimurium LT2 *iscR::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 ($OD_{600}$ = 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 ($Ap^{100}$), 30 μg/ml chloramphenicol ($Cm^{30}$), 50 μg/ml kanamycin ($Kn^{50}$), 20 μg/ml tetracycline ($Tc^{20}$), 1 μg/ml mitomycin C, 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), 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 promoter; (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. $mrr^{T518C}$) could be found in which a T518C mutation (resulting in a V173A substitution in the Mrr protein) abolished HP activation of the resulting $Mrr^{V173A}$ 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/$Mod^{LT2}$ antagonism (22). From this screen, one *mrr* allele (i.e. $mrr^{C835T}$) could be found in which a C853T mutation (resulting in a H279Y substitution in the Mrr protein) compromised HhaII-mediated activation of the resulting $Mrr^{H279Y}$ 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 $P_{BAD}$ promoter and was constructed by digesting a PCR amplicon of *mrr* (obtained using primers 5′-ATCGCTGCAGACG GTTCCTACCTATGAC-3′and 5′-CGATAAGCTTGCG TTTGCGGGGTTGAGG-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*:: $mrr^{T518C}$ and pBAD-*gfp*:: $mrr^{C835T}$ were constructed in the same fashion and encode Gfp-$Mrr^{V173A}$ and Gfp-$Mrr^{H279Y}$ 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 $P_{BAD}$ promoter and was constructed by digesting a PCR amplicon of *mrr* (obtained using primers 5′-ACTGCTGCAGTGATAAAGCTGCA AGGATGTACTATGACGG-3′ and 5′-CGATAAGCTT GCGTTTGCGGGGTTGAGG-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-$mod^{ED1a}$ encodes the *E. coli* ED1A type III Mod MTase [i.e. $Mod^{ED1A}$; (30)] under control of the IPTG-inducible $P_{trc}$ promoter and was constructed by digesting a PCR amplicon of the $mod^{ED1A}$ gene (obtained using primers 5′-ACGCTCTAGAGATACAGATACGT GGAA-3′ and 5′-CGTACTGCAGCATCTCATTCCCT TACAG-3′ on *E. coli* ED1A genomic DNA) with XbaI and PstI, before ligation in pTRC99A (31) digested with the same enzymes.

Plasmid pTRC-$mod^{LT2}$ encodes the *S.* Typhimurium LT2 type III (*Sty*LTI) Mod MTase [i.e. $Mod^{LT2}$; (32)] under control of the IPTG-inducible $P_{trc}$ promoter and was constructed by digesting a PCR amplicon of

the $mod^{LT2}$ gene (obtained using primers 5′-AAGTTCTA GAGGAGCGAAGATATCATGTTG-3′ and 5′-CGTAA AGCTTATCATCCCCTCTTAATAACG-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 $P_{trc}$ promoter and was constructed by digesting a PCR amplicon of *hhaII* [obtained using primers 5′-ACG CTCTAGAAGACAATATTATGTTTTC-3′ and 5′-CGT ACTGCAGCGTAACGCATTACTAATC-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 *in vitro* (data not shown), further supporting wild-type functionality of the expressed HhaII protein.

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

Plasmids were transformed to the appropriate bacterial strains via electroporation, and induction of the $P_{BAD}$ or $P_{trc}$ 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×*g*, 5 min) and resuspended in an equal volume of fresh prewarmed LB. Subsequently, a 100 µl 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 (HPIU-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 $P_{BAD}$ 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 about 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–

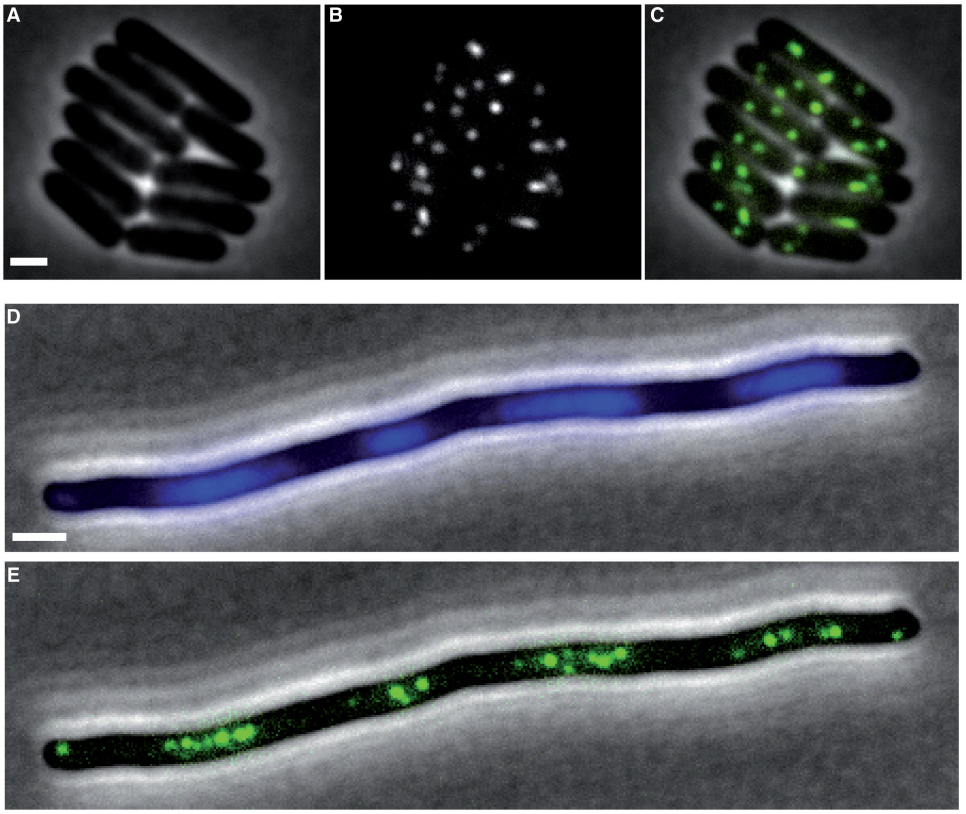


**Figure 1.** (**A–C**) Localization of Gfp-Mrr in unstressed MG1655 *mrr::Kn* pBAD-*gfp::mrr* cells (induced with 0.02% arabinose). Consecutive panels correspond to (A) phase contrast, (B) Gfp and (C) merged images of the same cells. (**D**, **E**) Localization of Gfp-Mrr and the nucleoid (stained with DAPI) in an MG1655 *lon* pBAD-*gfp::mrr* cell (induced with 0.02% arabinose) grown for 1 h in the presence of mitomycin C (1 μg/ml). The phase contrast image is merged with either the (D) DAPI or (E) Gfp image. The scale bar corresponds to 1 μm.

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 ($Mod^{ED1A}$) and *S.* Typhimurium LT2 ($Mod^{LT2}$) (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 $Mod^{ED1A}$ and $Mod^{LT2}$ 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/

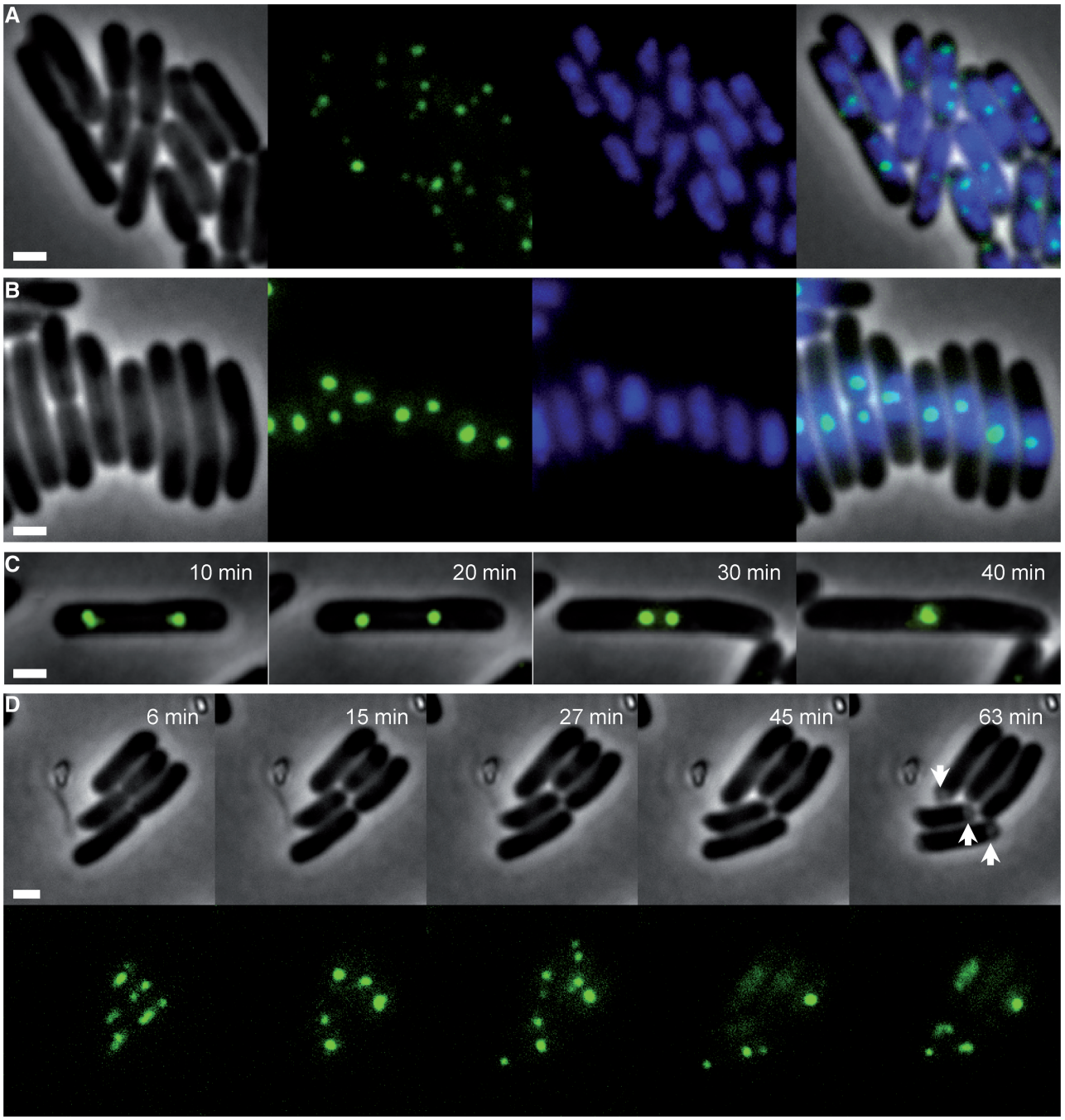


**Figure 2.** (**A**, **B**) Localization of Gfp-Mrr and the nucleoid (stained with DAPI) in (A) unstressed or (B) HP-stressed MG1655 *mrr::Kn* pBAD-*gfp::mrr* cells (induced with 0.02% arabinose). Consecutive panels correspond to phase contrast, Gfp, DAPI and merged images of the same cells. (**C**) 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.

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.

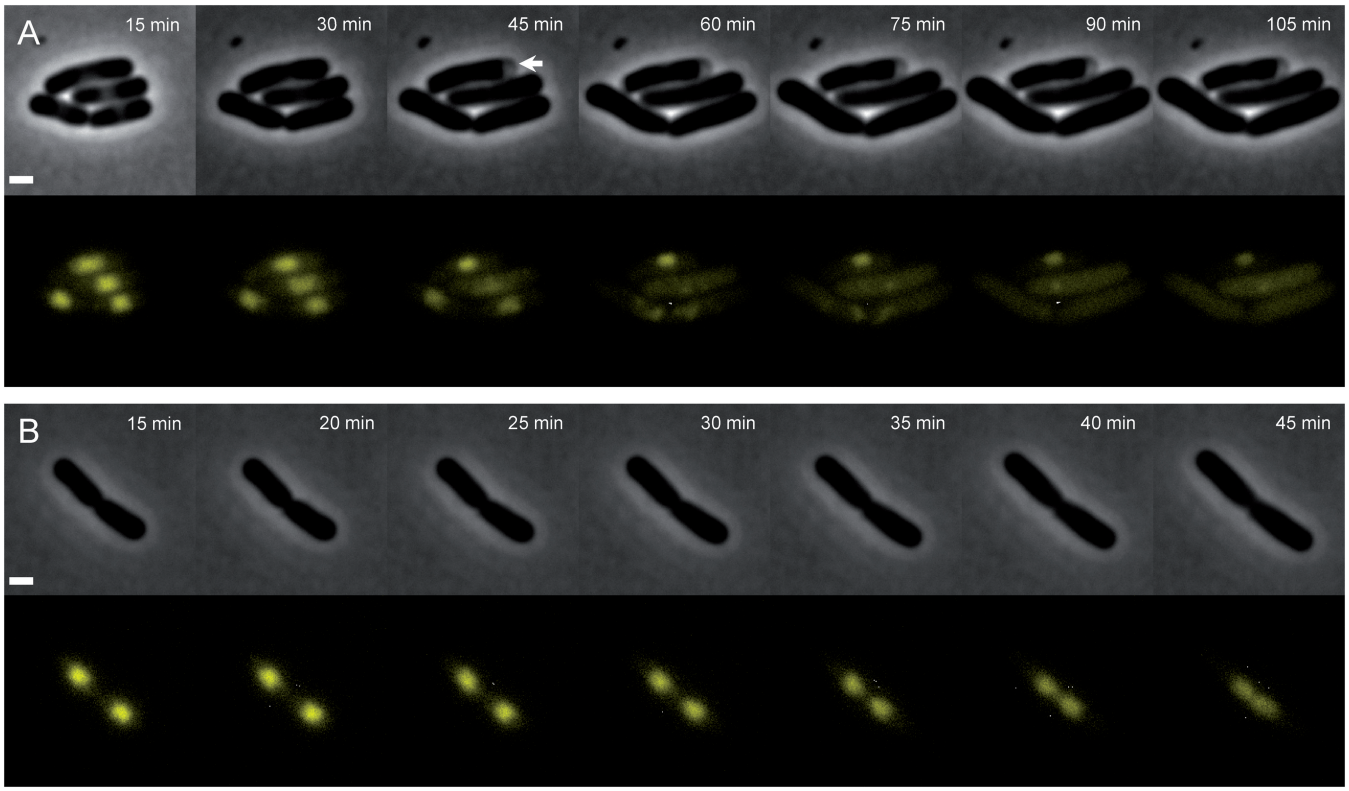


**Figure 3.** Nucleoid dynamics (**A**) in MG1655 *mrr::Tc iscR-yfp* pACYC184-$P_{BAD}$-*mrr* cells or (**B**) in a nearly divided MG1655 *mrr::Tc iscR-yfp* pACYC184-$P_{BAD}$-*mrr* cell at indicated time points after HP exposure. Phase contrast (upper panel) and Yfp (lower panel) images are shown. White arrows indicate when and where cells suffer from blebbing. The scale bar corresponds to 1 μm.

### 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. $Mrr^{V173A}$ 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

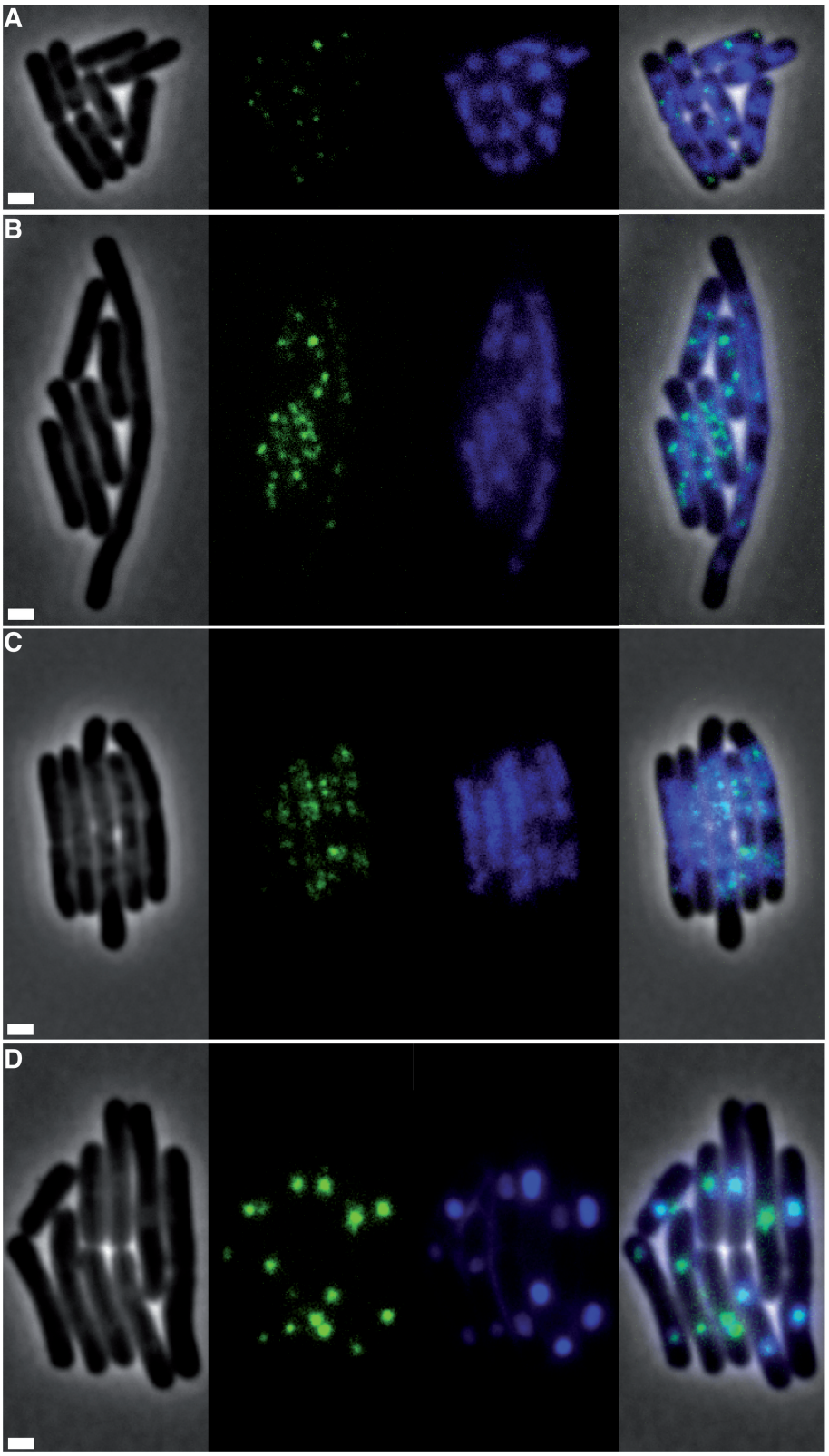


**Figure 4.** Localization of Gfp-Mrr and the nucleoid (stained with DAPI) in MG1655 *mrr::Kn* pBAD-*gfp::mrr* cells expressing (**A**) no foreign MTase (from empty pTRC99A), (**B**) the $Mod^{ED1A}$ MTase (from pTRC-$mod^{ED1A}$), (**C**) the $Mod^{LT2}$ MTase (from pTRC-$mod^{LT2}$) or (**D**) the 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.

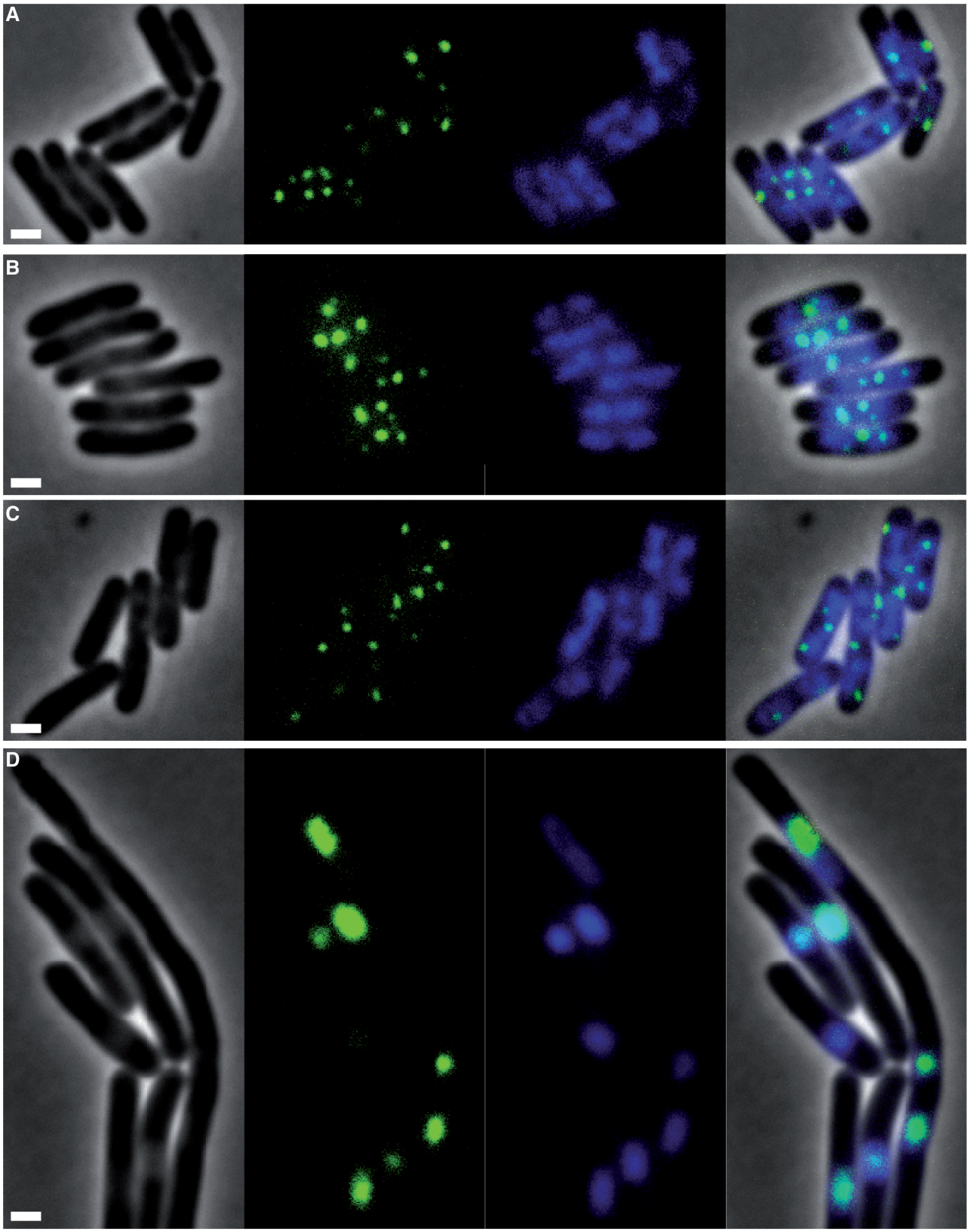


**Figure 5.** Localization of the Gfp-Mrr$^{V173A}$ variant and the nucleoid (stained with DAPI) in (**A**) unstressed or (**B**) HP-stressed MG1655 *mrr::Kn* pBAD-*gfp::mrr*$^{T518C}$ cells, or in MG1655 *mrr::Kn* pBAD-*gfp::mrr*$^{T518C}$ cells expressing (**C**) no foreign MTase (from empty pTRC99A) 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 μm.

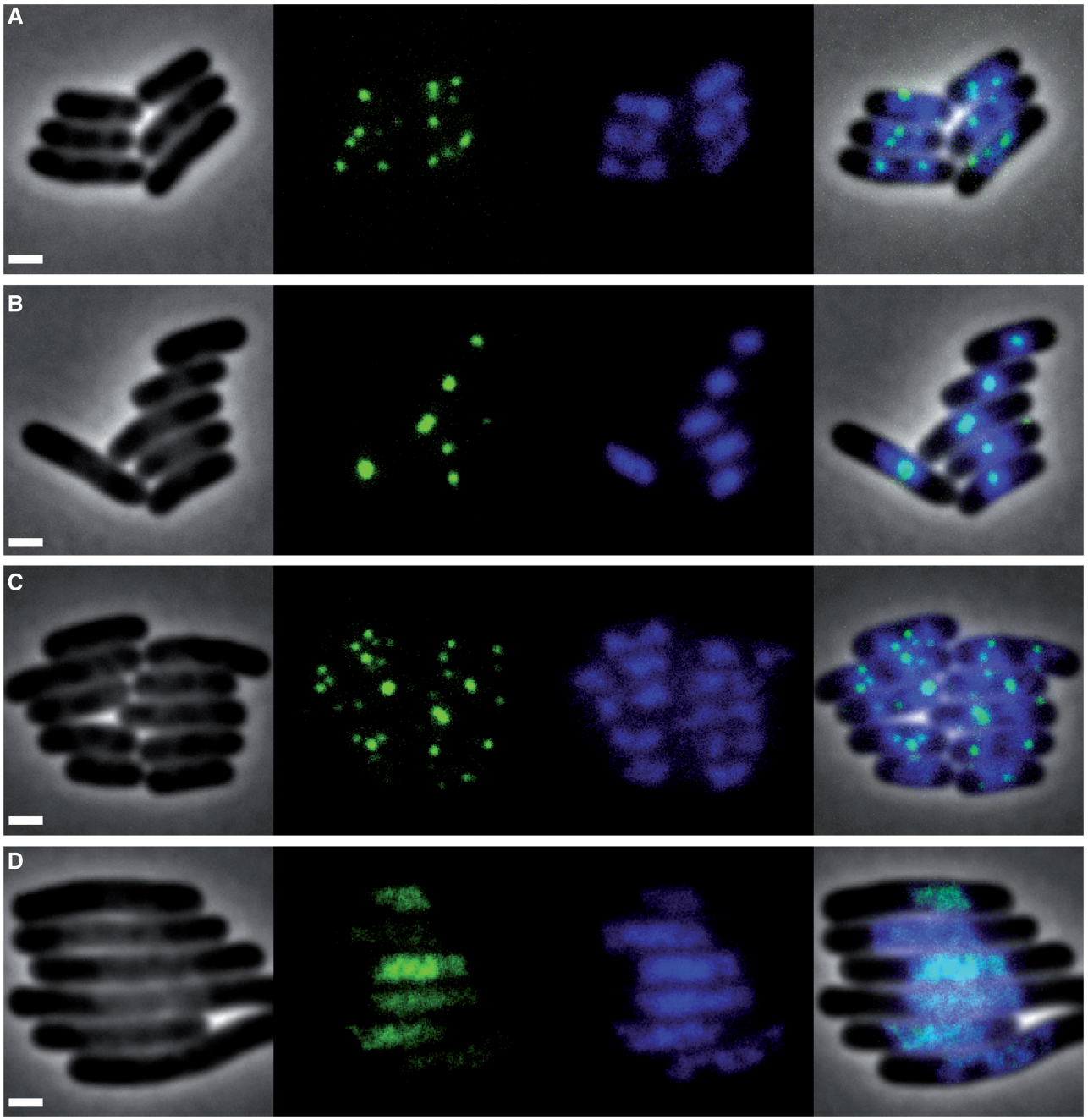


**Figure 6.** Localization of the Gfp-Mrr$^{H279Y}$ variant and the nucleoid (stained with DAPI) in (**A**) unstressed or (**B**) HP-stressed MG1655 *mrr::Kn* pBAD-*gfp::mrr*$^{C835T}$ cells, or in MG1655 *mrr::Kn* pBAD-*gfp::mrr*$^{C835T}$ cells expressing (**C**) no foreign MTase (from empty pTRC99A) 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.

(9,15,22). 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

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 near-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. $Mod^{ED1A}$ and $Mod^{LT2}$)-dependent or HhaII-dependent methylation. In sharp contrast to HhaII/Mrr (and HP/Mrr) activation, $Mod^{ED1A}$/Mrr and $Mod^{LT2}$/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 $Mod^{ED1A}$ or $Mod^{LT2}$ 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 $Mod^{ED1A}$/Mrr and $Mod^{LT2}$/Mrr activation, the displacement of the $Mrr^{H279Y}$ 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 *Sty*LTI MTase represented by $Mod^{LT2}$ being 5′-CAGAT-3′; (45)], the more generic and palindromic 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.

## FUNDING

This work was supported by doctoral fellowships from the Erasmus Mundus External Cooperation Window (EMECW13-Eurindia; to A.G.) and the Flemish Agency for Innovation by Science and Technology (IWT-Vlaanderen; to I.P.), and grants from the KU Leuven Research Fund (STRT1/10/036, IDO/10/012, DBOF/12/035 and GOA 2011/03), the Research Foundation of Flanders (FWO-Vlaanderen; G.0580.11), the Flemish government (CASAS METH/08/04) and the Hercules foundation (HER/08/21, and HER/08/39). Funding for open access charge: KU Leuven Research Fund (STRT1/10/036 and IDO/10/012).

*Conflict of interest statement*. None declared.

## REFERENCES

1. Roberts,R.J., Belfort,M., Bestor,T., Bhagwat,A.S., Bickle,T.A., Bitinaite,J., Blumenthal,R.M., Degtyarev,S.K., Dryden,D.T.F., Dybvig,K. *et al*. (2003) A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res*., **31**, 1805–1812.
2. Loenen,W.A.M. and Raleigh,E.A. (2013) The other face of restriction: modification-dependent enzymes. *Nucleic Acids Res*. (epub ahead of print).
3. Revel,H.R. (1967) Restriction of nonglucosylated T-even bacteriophage: properties of permissive mutants of *Escherichia coli* B and K12. *Virology*, **31**, 688–701.
4. Raleigh,E.A. and Wilson,G. (1986) *Escherichia coli* K-12 restricts DNA containing 5-methylcytosine. *Proc. Natl. Acad. Sci. USA*, **83**, 9070–9074.
5. Rifat,D., Wright,N.T., Varney,K.M., Weber,D.J. and Black,L.W. (2008) Restriction endonuclease inhibitor IPI* of bacteriophage T4. *J. Mol. Biol*., **375**, 720–734.
6. Fukuda,E., Kaminska,K.H., Bujnicki,J.M. and Kobayashi,I. (2008) Cell death upon epigenetic genome methylation: a novel function of methyl-specific deoxyribonucleases. *Genome Biol*., **9**, R163.
7. Ishikawa,K., Fukuda,E. and Kobayashi,I. (2010) Conflicts targeting epigenetic systems and their resolution by cell death: novel concepts for methyl-specific and other restriction systems. *DNA Res*., **17**, 325–342.

8. Ravi,R.S., Sozhamannan,S. and Dharmalingam,K. (1985) Transposon mutagenesis and genetic mapping of the *rglA* and *rglB* loci of *Escherichia coli*. *Mol. Gen. Genet*., **198**, 390–392.
9. Heitman,J. and Model,P. (1987) Site-specific methylases induce the SOS DNA repair response in *Escherichia coli*. *J. Bacteriol*., **169**, 3243–3250.
10. Raleigh,E.A., Trimarchi,R. and Revel,H. (1989) Genetic and physical mapping of the *mcrA* (*rglA*) and *mcrB* (*rglB*) loci of *Escherichia coli* K-12. *Genetics*, **122**, 279–296.
11. Kelleher,J.E. and Raleigh,E.A. (1991) A novel activity in *Escherichia coli* K-12 that directs restriction of DNA modified at CG dinucleotides. *J. Bacteriol*., **173**, 5220–5223.
12. Sibley,M.H. and Raleigh,E.A. (2004) Cassette-like variation of restriction enzyme genes in *Escherichia coli* C and relatives. *Nucleic Acids Res*., **32**, 522–534.
13. Waite-Rees,P.A., Keating,C.J., Moran,L.S., Slatko,B.E., Hornstra,L.J. and Benner,J.S. (1991) Characterisation and expression of the *Escherichia coli* Mrr restriction system. *J. Bacteriol*., **173**, 5207–5219.
14. Aertsen,A., Van Houdt,R., Vanoirbeek,K. and Michiels,C.W. (2004) An SOS response induced by high pressure in *Escherichia coli*. *J. Bacteriol*., **186**, 6133–6141.
15. Aertsen,A. and Michiels,C.W. (2005) Mrr instigates the SOS response after high-pressure stress in *Escherichia coli*. *Mol. Microbiol*., **58**, 1381–1391.
16. Aertsen,A., Faster,D. and Michiels,C.W. (2005) Induction of Shiga toxin-converting prophage in *Escherichia coli* by high hydrostatic pressure. *Appl. Environ. Microbiol*., **71**, 1155–1162.
17. Aertsen,A. and Michiels,C.W. (2005) SulA-dependent hypersensitivity to high pressure and hyperfilamentation after high-pressure treatment of *Escherichia coli lon* mutants. *Res. Microbiol*., **156**, 233–237.
18. Ghosh,A. and Aertsen,A. (2013) Cellular filamentation after sublethal High-Pressure shock in *Escherichia coli* K12 is Mrr Dependent. *Curr. Microbiol*., **67**, 522–524.
19. Ivancic-Bace,I., Vlasic,I., Cogelja-Cajo,G., Brcic-Kostic,K. and Salaj-Smic,E. (2006) Roles of PriA protein and double-strand DNA break repair functions in UV-induced restriction alleviation in *Escherichia coli*. *Genetics*, **174**, 2137–2149.
20. Blakely,G.W. and Murray,N.E. (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.
21. Aertsen,A. and Michiels,C.W. (2006) Upstream of the SOS response: figure out the trigger. *Trends Microbiol*., **14**, 421–423.
22. Tesfazgi Mebrhatu,M., Wywial,E., Ghosh,A., Michiels,C.W., Lindner,A.B., Taddei,F., Bujnicki,J.M., Van Melderen,L. and Aertsen,A. (2011) Evidence for an evolutionary antagonism between Mrr and Type III modification systems. *Nucleic Acids Res*., **39**, 5991–6001.
23. Blattner,F.R., Plunkett,G. III, Bloch,C.A., Perna,N.T., Burland,V., Riley,M., Collado-Vides,J., Glasner,J.D., Rode,C.K., Mayhew,G.F. *et al*. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science*, **277**, 1453–1462.
24. Kleckner,N., Bender,J. and Gottesman,S. (1991) Uses of transposons with emphasis on Tn10. *Methods Enzymol*., **204**, 139–180.
25. Datsenko,K.A. and Wanner,B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA*, **97**, 6640–6645.
26. Cherepanov,P.P. and Wackernagel,W. (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene*, **158**, 9–14.
27. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York, NY.
28. Aertsen,A., Tesfazgi Mebrhatu,M. and Michiels,C.W. (2008) Activation of the *Salmonella typhimurium* Mrr protein. *Biochem. Biophys. Res. Commun*., **367**, 435–439.
29. Hallez,R., Geeraerts,D., Sterckx,Y., Mine,N., Loris,R. and Melderen,L.V. (2010) New toxins homologous to ParE belonging to three-component toxin–antitoxin systems in *Escherichia coli* O157:H7. *Mol. Microbiol*., **76**, 719–732.
30. Touchon,M., Hoede,C., Tenaillon,O., Barbe,V., Baeriswyl,S., Bidet,P., Bingen,E., Bonacorsi,S., Bouchier,C., Bouvet,O. *et al*. (2009) Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. *PLoS Genet*., **5**, e1000344.
31. Amann,E., Ochs,B. and Abel,K.J. (1988) Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene*, **69**, 301–315.
32. McClelland,M., Sanderson,K.E., Spieth,J., Clifton,S.W., Latreille,P., Courtney,L., Porwollik,S., Ali,J., Dante,M., Du,F. *et al*. (2001) Complete genome sequence of *Salmonella enterica* serovar *typhimurium* LT2. *Nature*, **413**, 852–856.
33. Schoner,B., Kelly,S. and Smith,H.O. (1983) The nucleotide sequence of the HhaII restriction and modification genes from *Haemophilus haemolyticus*. *Gene*, **24**, 227–236.
34. Cenens,W., Mebrhatu,M.T., Makumi,A., Ceyssens,P.J., Lavigne,R., Van Houdt,R., Taddei,F. and Aertsen,A. (2013) Expression of a novel P22 ORFan gene reveals the phage carrier state in *Salmonella typhimurium*. *PLoS Genet*., **9**, e1003269.
35. Melderen,L.V. and Aertsen,A. (2009) Regulation and quality control by Lon-dependent proteolysis. *Res. Microbiol*., **160**, 645–651.
36. Berlatzky,I.A., Rouvinski,A. and Ben-Yehuda,S. (2008) Spatial organization of a replicating bacterial chromosome. *Proc. Natl. Acad. Sci. USA*, **105**, 14136–14140.
37. Elez,M., Murray,A.W., Bi,L.J., Zhang,X.E., Matic,I. and Radman,M. (2010) Seeing mutations in living cells. *Curr. Biol*., **20**, 1432–1437.
38. Wang,W., Li,G.W., Chen,C., Xie,X.S. and Zhuang,X. (2011) Chromosome organization by a nucleoid-associated protein in live bacteria. *Science*, **333**, 1445–1449.
39. Dame,R.T., Kalmykowa,O.J. and Grainger,D.C. (2011) Chromosomal macrodomains and associated proteins: implications for DNA organization and replication in Gram negative bacteria. *PLoS Genet*., **7**, e1002123.
40. Badrinarayanan,A., Reyes-Lamothe,R., Uphoff,S., Leake,M.C. and Sherratt,D.J. (2012) *In vivo* architecture and action of bacterial structural maintenance of chromosome proteins. *Science*, **338**, 528–531.
41. Dorman,C.J. (2013) Genome architecture and global gene regulation in bacteria: making progress towards a unified model? *Nat. Rev. Microbiol*, **11**, 349–355.
42. Jin,D.J., Cagliero,C. and Zhou,Y.N. (2013) Role of RNA polymerase and transcription in the organization of the bacterial nucleoid. *Chem. Rev*., **113**, 8662–8682.
43. Smith,B.T., Grossman,A.D. and Walker,G.C. (2002) Localization of UvrA and effect of DNA damage on the chromosome of *Bacillus subtilis*. *J. Bacteriol*., **184**, 488–493.
44. Loenen,W.A., Dryden,D.T., Raleigh,E.A., Wilson,G.G. and Murray,N.E. (2013) Highlights of the DNA cutters: a short history of the restriction enzymes. *Nucleic Acids Res*. (epub ahead of print).
45. De Backer,O. and Colson,C. (1991) Identification of the recognition sequence for the M.StyLTI methyltransferase of *Salmonella typhimurium* LT7: an asymmetric site typical of type-III enzymes. *Gene*, **97**, 103–107.
46. Mann,M.B., Rao,R.N. and Smith,H.O. (1978) Cloning of restriction and modification genes in *E. coli*: the HbaII system from *Haemophilus haemolyticus*. *Gene*, **3**, 97–112.
47. Yitzhaki,S., Rostron,J.E., Xu,Y., Rideout,M.C., Authement,R.N., Barlow,S.B. and Segall,A.M. (2012) Similarities between exogenously- and endogenously-induced envelope stress: the effects of a new antibacterial molecule, TPI1609-10. *PLoS One*, **7**, e44896.