DNA Research, 2020, 0(0), 1–13
doi: 10.1093/dnares/dsaa003
Advance Access Publication Date: 13 March 2020

OXFORD

Low-level expression of the Type II restriction–modification system confers potent bacteriophage resistance in *Escherichia coli*

Karolina Wilkowska, Iwona Mruk , Beata Furmanek-Blaszk, and Marian Sektas *

Department of Microbiology, Faculty of Biology, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland

*To whom correspondence should be addressed. Tel. +4858 5236068. Fax. +4858 5236073. Email: marian.sektas@ug.edu.pl

Received 29 October 2019; Editorial decision 8 March 2020; Accepted 9 March 2020

Abstract

Restriction–modification systems (R–M) are one of the antiviral defense tools used by bacteria, and those of the Type II family are composed of a restriction endonuclease (REase) and a DNA methyltransferase (MTase). Most entering DNA molecules are usually cleaved by the REase before they can be methylated by MTase, although the observed level of fragmented DNA may vary significantly. Using a model EcoRI R–M system, we report that the balance between DNA methylation and cleavage may be severely affected by transcriptional signals coming from outside the R–M operon. By modulating the activity of the promoter, we obtained a broad range of restriction phenotypes for the EcoRI R–M system that differed by up to 4 orders of magnitude in our biological assays. Surprisingly, we found that high expression levels of the R–M proteins were associated with reduced restriction of invading bacteriophage DNA. Our results suggested that the regulatory balance of cleavage and methylation was highly sensitive to fluctuations in transcriptional signals both up- and downstream of the R–M operon. Our data provided further insights into Type II R–M system maintenance and the potential conflict within the host bacterium.

Key words: DNA restriction, promoter, restriction–modification system expression, SOS response, transcription regulation

1. Introduction

Restriction–modification (R–M) systems are an essential part of the bacterial defense against invading DNA.[1–5] The components of these systems, an endonuclease (REase) and a methyltransferase (MTase), work to either cleave or protect DNA in a sequence-specific manner. Given their opposing roles, it is reasonable to postulate that gene expression or other biochemical mechanisms have evolved to balance the activities of the MTase and the REase inside a host.[6–12] Maintenance of an optimal balance is crucial for the integrity of the genome, the survival of the cell and the stability of the R–M system within a host. To avoid methylation of the incoming DNA before its restriction, it is essential that a potent anti-phage defense system is well established with optimal regulation within a narrow window of flexibility. The relationships between the strength of the antiviral defense and the cellular level of R–M protein production remain to be fully characterized. Regulatory control of R–M system gene expression favors relatively low levels of REase and MTase,[13] and the REase is often under additional controls (dedicated controlling protein, regulatory MTase, or antisense RNAs) to prevent becoming toxic to the host.[9]

The role of the R–M system in the context of a cell is not fully defined, and little is known about the possible impacts of aberrant DNA cleavage on host genome integrity. There seem to be critical moments during the cell cycle; otherwise, some regions of the host chromosome may become vulnerable to residual REase.[12,14–17] R–M systems can be horizontally transferred between various genomes,[18–22] and the effects of unbalanced expression are especially dramatic when the newly acquired R–M system is being established in a host

with unprotected genome.[20,23–28] Cell death during this period remains high unless the R–M system gene expression regulation reaches a fine-tuned balance facilitating survival. Proper balance is also critical for cell vitality during the loss of R–M genes, as the gradual decrease in modification activity leaves the chromosome exposed to restriction. This effect is lethal for the majority of cells.[15,16,29–31] It is, therefore, not surprising that some R–M gene complexes are recognized as self-mobilizable elements.[31–33]

In this study, we used the EcoRI R–M system of *Escherichia coli* as a model[34–38] to study the relationship between *ecoRIR* and *ecoRIM* expression levels and test the factors of the optimal balance of their enzymatic activities. Originally, the genes of the EcoRI R–M system were discovered on a pMB1 plasmid located close to the highly site-specific recombinogenic *cer* site. Following the concerted action of XerCD and other accessory proteins, this region might serve as the multimer resolution crossover site for many *cer*-like sequence homologues.[39–42] This region also contributes to Xer-independent dimer formation, but at very low frequencies.[43] Previous studies reported that EcoRI R–M systems carried by ColE1-type plasmid derivatives are sometimes functionally unstable and result in a variety of R–M system phenotypes.[44–47] Notably, the phenotypic alterations of the EcoRI R–M systems could not be attributed to simple insertion or deletion changes and the actual mechanism responsible for the observed conversion remains unknown (e.g. phenotypically cryptic restriction activity, which is not functional *in vivo* but is detectable after extraction from the same cells).[45] Reported here analysis of the EcoRI R–M system in several plasmid backgrounds carried on P15A replicon derivatives[48] revealed a range of restriction activity phenotypes (high, low, and completely abolished). Our results have provided a framework for understanding the previously observed phenotypic instabilities, by linking the disruption of the restriction/modification balance to changes in R–M operon gene expressions that appear to be the result of the R–M system's close proximal genetic context. We propose that shaping of the functional diversity of R–M systems may likely be a consequence of the cycles of establishment events that occur in new hosts once the genes have been horizontally transferred. Our data provided further insights into Type II R–M system maintenance and the potential conflict within the host bacterium.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli K-12 strains used in this work are presented in Table 1. *Escherichia coli* cells were grown in LB medium with antibiotics at the following concentrations when necessary: ampicillin (Ap) at 100 μg/ml, chloramphenicol (Cm) at 30 μg/ml, and tetracycline (Tc) 15 μg/ml. Isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich, USA) and L-arabinose (Sigma-Aldrich) were used as indicated in the experiments. Plasmids were introduced into chemically competent cells by a standard procedure.[49] Their relevant features are presented in Supplementary Table S1 as well as information on their construction using oligonucleotides in Supplementary Table S2.

2.2. Growth competition assay

Escherichia coli MG1655 strains bearing or lacking the EcoRI R–M system were grown from single colonies. To quantitate the initial number of bacteria (CFU/ml) used for inoculation of a mixed culture, each strain was serially diluted and spread on LB plates. Each pair of the examined strains (MG1655, as a negative control without any Type II R–M systems) was also mixed at a 1:1 cell number ratio and diluted 1:50 into 10 ml of fresh LB without antibiotics. Each overnight co-culture (16–18 h at 37 °C) was then diluted 10^4 into fresh LB medium. The cycle of consecutive dilutions and growth of mixed cultures was repeated twice. Finally, each mixed competition culture was sampled, diluted, and spread quantitatively onto LB agar containing chloramphenicol and onto antibiotic-free LB agar plates. The colonies were counted and the ratio of cells carrying plasmids to the total number of cells was determined for each of the tested co-cultures.

2.3. Phage restriction assay

The restriction activity of *E. coli* cells carrying the *ecoRIR* gene was measured by determining the efficiency of plating of phage λ_{vir}, which has four EcoRI sites[50] and is unable to form lysogens. All experiments were conducted with freshly transformed cells. Serial dilutions of λ_{vir} phage in TM (10 mM $MgSO_4$ and 100 mM NaCl) buffer were prepared from 1 to 10^8 cells. In the qualitative assay, 3 ml top agar preheated to 44 °C with 300 μl bacterial culture was poured on the LA bottom layer. Ten microliters of each serial dilution of a phage lysate was then spotted onto the top of the agar and incubated overnight at 37 °C. The quantitative assay involved a mixture of the appropriate phage dilution (100 μl) and host bacteria (10–300 μl, at $OD_{600} = 0.6$) and was incubated for 20 min at room temperature, then 3 ml of the top agar was added, mixed, and then poured onto the LA bottom layer. The plates were incubated overnight at 37 °C. The efficiency of plaque formation was calculated as the number of plaque-forming units (PFUs) obtained on bacteria with the tested plasmid divided by the number of PFUs on bacteria with the pACYC184 plasmid. Relative restriction (=1) denoted the EcoRI R–M system carried out on the pIM-RM plasmid.

2.4. Quantitative real-time PCR analysis

Three milliliters of *E. coli* ER1992 cells containing EcoRI R–M plasmids was harvested during exponential growth. The cells were centrifuged, then the bacterial pellet was resuspended in 1 ml of StayRNA reagent to prevent RNA degradation (A&A Biotechnology, Gdynia, Poland). The total cellular RNA was extracted using the Total RNA Mini Plus Kit (A&A Biotechnology) according to the manufacturer's instructions. After elution, the RNA was treated with DNase I for 60 min at 37 °C (Thermo Fisher Scientific, Waltham, MA, USA) and then at 65 °C for 15 min to inactivate the enzyme. The RNA was then used as a template for first-strand cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Three sets of primers (Supplementary Table S2) were used to estimate transcripts levels of *ecoRIR*, *ecoRIM*, and a housekeeping gene (16S *rrn*). Primers were designed to avoid regions involved in regulatory circuits.[35,36] Each qPCR reaction (10 μl) contained 5 μl SG qPCR Master Mix (2×) with SYBR Green I fluorescent dye, Perpetual Taq DNA polymerase, and dNTPs (Eurx, Gdansk, Poland), 3 μl H_2O, 1 μl of 5 μM forward and reverse primer mix, and 1 μl of diluted cDNA as a template. The qPCR was conducted using a Roche Lightcycler (Roche, Basel, Switzerland) using the following conditions: a predenaturation step at 94 °C for 3 min and 35 cycles at 94 °C for 30 s, annealing at 50 °C for 30 s, and polymerization at 72 °C for 20 s. The expected product lengths were 172, 170, and 170 bases for *ecoRIR*, *ecoRIM*, and 16S *rrn*, respectively. Biological triplicates were taken for each sample and the qPCR was independently repeated at least twice. Melting curve analysis was conducted to confirm the formation of the expected products. The quantitative model included the PCR efficiency for each cDNA and

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

Name	Relevant genotype/features	Source
AB1157	*thr-1, ara-14, leuB6,* Δ(*gpt-proA*)*62, lacY1, tsx-33, supE44, galK2, rac−, hisG4*(*Oc*)*, rfbD1, mgl-51, rpsL31, kdgK51, xyl-5, mtl-1, argE3* (*Oc*)*, thi-1, qsr,−*	Ref. 97
DH5α	(F$^-$ λ^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ*80dlacZ*Δ*M15* Δ(*lacZYA-argF*)*U169, hsdR17*($r_K^- m_K^+$)	New England Biolabs, Ipswich, USA
MG1655	RecA$^+$ prototrophic strain, that carried no Type II R–M systems but does produce the Type I system EcoKI	Ref. 98
ER1992	F$^-$ Δ(*argF-lac*)*U169 supE44 e14*$^-$ *dinD1::Mu dI1734* (Km^r, LacZ$^+$) *endA1 thi-1* Δ(*mcrC-mrr*)*114::IS10*	Ref. 48 Ref. 99
ER1992E	Host with a chromosomal encoded EcoRI R–M system at the *attB* locus	Ref. 14
SS996	(P_{sulA}::*gfp*), SOS reporter	Ref. 100
MP060	(MG1655 ΔattHK022::P_{sulA}-*yfp*), SOS reporter	Ref. 88
MP064	(MG1655, ΔattHK022::P_{sulA}-*yfp* Δ*recA*) SOS reporter	Ref. 88
DS956	(AB1157 *recF lacI*^{q} *lacZ*Δ*M15 argRA9::fol* Tp^r) Xer/*cer* defective	Ref. 101
DS957	(AB1157 *recF lacI*^{q} *lacZ*Δ*M15 pepA*::Tn*5* Km^r), Xer/*cer* defective	Ref. 102
DS981	(AB1157 *recF lacI*^{q} *lacZ*Δ*M15 xer*C Km^r), Xer/*cer* defective	Ref. 103
DS9008	(AB1157 *recF lacI*^{q} *lacZ*Δ*M15 xerD2*::Tn*10-9* Km^r), Xer/*cer* defective	Ref. 104

was obtained by serially diluting it and then plotting the Ct values against cDNA input to generate a slope.[51,52] We observed high linearity ($R^2 > 0.98$) for all measurements.

3. Results

We noticed a substantial variation in DNA restriction, using a λ phage plaque assay in the same *E. coli* host strain that carried several EcoRI R–M plasmids (Fig. 1A). The EcoRI R–M system located on the pACYCeco plasmid showed significantly higher phage restriction (similar to the pMB1-related NTP14 plasmid),[47] in comparison to the system carried by the pIM-RM plasmid of the same P15A replicon (Supplementary Fig. S1).[35,53] The differences were ∼1,300-fold between these two plasmids, and 1,900-fold comparing the plasmid and chromosomal *ecoRIRM* locations (Fig. 1A). Notably, the nucleotide sequences of both systems were identical, and the promoter regions, consisting of the main P_R promoter embedded within the binding elements of the Xer/*cer* recombination system and including P_{cer} promoter,[36,54–56] were also very close in sequence. The differences involved the orientation of the EcoRI R–M operon with respect to the chloramphenicol gene and the presence of a weak constitutive promoter P_L1 from the λ bacteriophage,[53,57] which was coupled to the λ *nutL* anti-terminator (Fig. 2). Western blot analysis of the REase and MTase for both strains showed that high expression of the R–M system was not correlated with the observed antiviral activity (Fig. 1A vs. 1B). Paradoxically, plasmid pACYCeco, which confers high relative restriction, produced much less REase (below the level of detection in this assay) than the systems located on the pIM-RM plasmid or on the control plasmid, where the EcoRI MTase and REase enzymes were overproduced upon arabinose induction (Fig. 1B). We then sought to understand the basis for this highly varied expression and the lack of correspondence between REase levels and degree of restriction.

3.1. The enhanced restriction phenotype was not due to N/*nutL* anti-termination

It was possible that R–M system expression may be affected during λ phage infection when the *N* anti-terminator protein was supplied, and this could cause for the high restriction pattern observed in the pACYCeco construct (*nutL* anti-terminator upstream of P_R) (Fig. 2). To address this possibility, we provided *N* gene expression from the arabinose-inducing promoter of the pBAD-N vector (Supplementary Table S1) and measured the relative level of β-galactosidase reporter inserted downstream of the *nutL* site (pACYClacZ) (Supplementary Table S1). We observed up to a 3.3-fold increase in β-galactosidase activity when the N protein was present (Fig. 1C), which was in agreement with other reports detailing the effects of N protein on transcription.[58,59]

We also used a time course to measure the kinetics of R.EcoRI production during λ phage infection when the N protein was supplied by its natural source. We found higher levels of R.EcoRI protein in the λ phage-infected bacteria when compared with the uninfected control cells (Fig. 1D). These results confirmed that REase levels were slightly higher when the anti-termination process was active, but the levels were still significantly lower than those produced in the pIM-RM background (Fig. 1C). We inactivated the weak t_L0 terminator accompanying the *nutL* region[58] in pACYCeco to produce pACYCΔnutL (Fig. 2; Supplementary Table S1), and, as expected, both the REase level (Fig. 1B, lane 5 vs. lane 2) and the relative restriction (phage assay, data not shown) remained unchanged. Consistent with this possibility was the observation of a 2-fold increase in LacZ activity when the reporter was cloned downstream of P_L1 (pACYCΔnutLacZ; Supplementary Table S1) relative to the *nutL*-t_L0 bearing pACYClacZ (Fig. 1C). These results indicated that the N/*nutL* elements were likely not responsible for the two distinct phenotypes observed for relative restriction (pACYCeco vs. pIM-RM).

3.2. Additional upstream transcription for the *Eco*RI operon was responsible for the difference in restriction efficiency

We next asked whether the observed differences in restriction efficiency might simply be due to varied *ecoRIRM* expressions in both plasmids. We designed *lacZ* translational fusions for the *ecoRIR* and *ecoRIM* genes, making sure that the EcoRI regulatory antisense RNAs were preserved.[35] For the *ecoRIM::lacZ* fusion, the REase gene was first mutated, avoiding transcriptional polarity effects, but preventing active REase from being produced. A restriction-deficient REase mutant (Lys113Ala) was generated as previously reported.[60] LacZ activity was measured for the two translational fusion constructs, *ecoRIM::lacZ* and *ecoRIR::lacZ* in both pIM-RM and

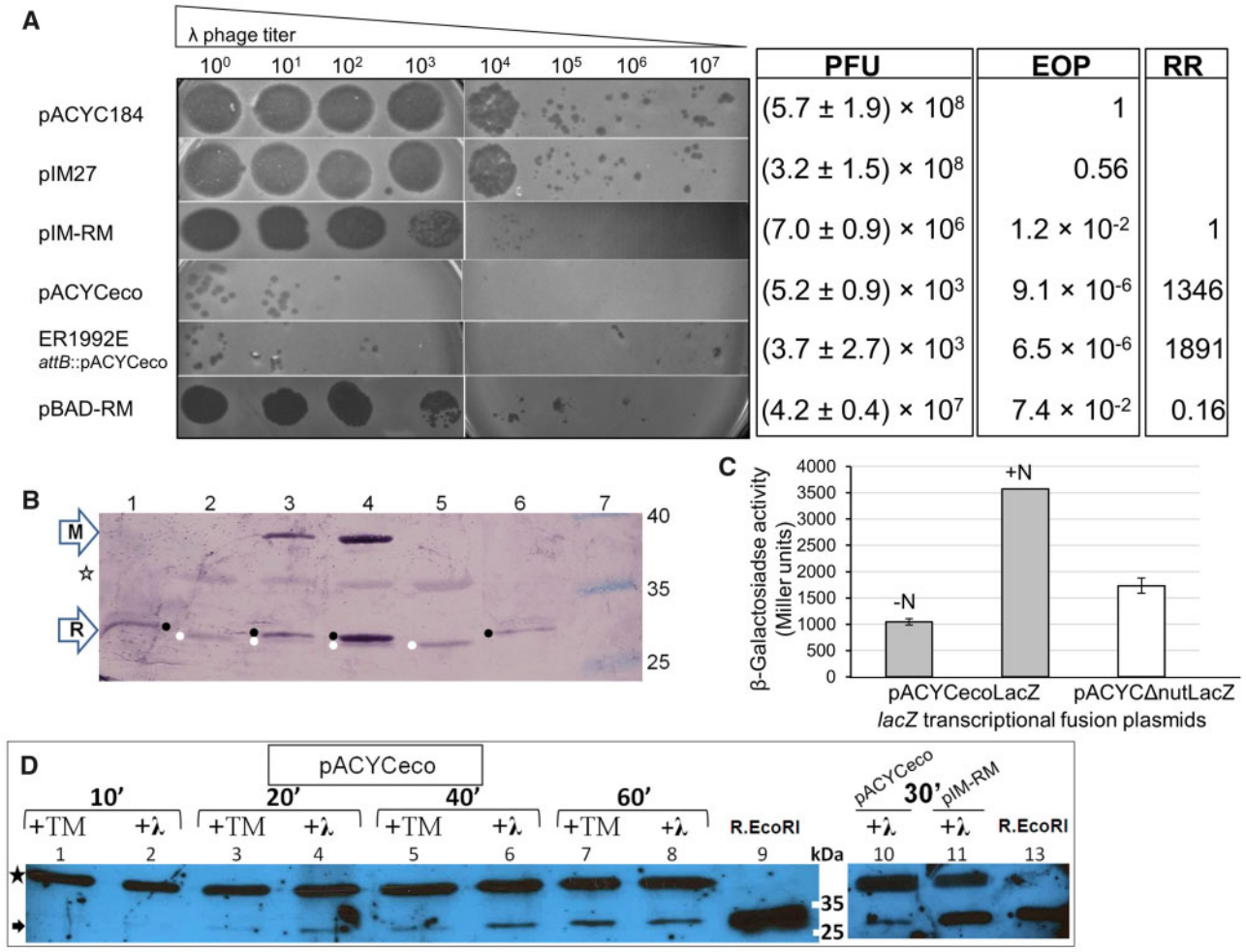

Figure 1. Phage restriction variation in *Escherichia coli* cells carrying the EcoRI R–M system measured as phage λ_{vir} infection efficiency. (A) The qualitative assay involved 5 μl of serial dilutions of $\lambda b2_{vir}$ phage, which were spotted onto LB agar plates with an *E. coli* ER1992 lawn carrying the indicated plasmids or no plasmids [ER1992E (EcoRI R–M)$^{+}$ strain] and incubated overnight at 37 °C. Quantitative results of the restriction activity of *E. coli* ER1992 strains. EOP, efficiency of plaques, PFUs tested on plasmid/strains divided by PFUs on pACYC184; and RR, restriction relative to the pIM-RM bearing strain. The standard deviation from at least three measurements is indicated. (B) Western blots of the lysates of the late exponential phase harvested MG1655 bacteria carrying: pACYCeco, pIM-RM, pBAD-RM, and pACYCΔnutL. Lanes R contain a purified R.EcoRI preparation. Lane 'sm' contains molecular size markers. The levels of R.EcoRI and M.EcoRI proteins in bacterial crude extracts were tested by western blotting using rabbit anti-M.EcoRI and anti-R.EcoRI polyclonal antibodies and visualized by BCIP/NBT (nitroblue tetrazolium) as the color development reagent. The star and white circles indicate the location of unknown antigen proteins. Note that expression of the EcoRI R–M system on pBAD-RM was induced using 0.04% L-arabinose for 2 h. (C) Relative strength of the P_{L1} promoter and *nutL*/N anti-termination in pACYCeco expressed as reporter LacZ activity. Error bars represent standard deviations from at least three independent experiments. (D) The anti-termination-mediated increase of *ecoRIR* expression from pACYCeco plasmid, by λ-N protein, 10, 20, 30, 40, and 60 min after infection. Note that the cell lysis was observed after 60 min of post-infection time (Line 8), thus the bacterial lysate might be non-representative. Also shown is the comparison of the expression levels of the *ecoRIRM* from pIM-RM after 30 min of λ_{vir} phage infection. The star indicates the location of the unknown antigen protein, and the arrow indicates the position of the R.EcoRI enzyme.

pACYCeco derivatives (Fig. 3A). The results for fusions under their constitutive expression showed that both derivatives of pIM-RM had ∼5-fold higher gene expression than the pACYCeco-derived plasmids (Fig. 3B; pIM-RMlacZR vs. pACYClacZR and pIM-RMlacZM vs. pACYClacZM). Quantitative RT-PCR of total RNA from cells carrying pACYCeco and pIM-RM was used to confirm the *ecoRIR* and *ecoRIM* transcript levels. As expected, higher levels (∼14-fold for *ecoRIR* and 10-fold for *ecoRIM*) of the EcoRI operon expression for pIM-RM were observed when compared with pACYCeco (Fig. 3C). Quantitative densitometric analysis revealed 2-fold molar excess of M.EcoRI protein over R.EcoRI in early stationary phase for cells carrying pIM-RM. In addition, we cloned the *araBAD* promoter upstream of the P_R promoter of the EcoRI operon (pACYCara plasmid; Fig. 2) to determine whether a strong upstream transcriptional signal might interfere with the two regulatory circuits responsible for the fine-tuned restriction/modification gene expression balance.[35,36] Such constructs with *lacZ* reporter fused to *ecoRIR* and *ecoRIM* (pACYCecoARAlacZR and pACYCecoARAlacZM, respectively) generated EcoRI operon overexpression after arabinose induction [Fig. 3A (in brackets) and B]. Our results with pACYCara-based expression clearly showed that high operon induction was associated with lower relative restriction (λ_{vir} plaque formation) (Figs 3B and 4B), confirming the earlier results obtained for pBAD-RM construct (Fig. 1A and B, Supplementary Fig. S2).

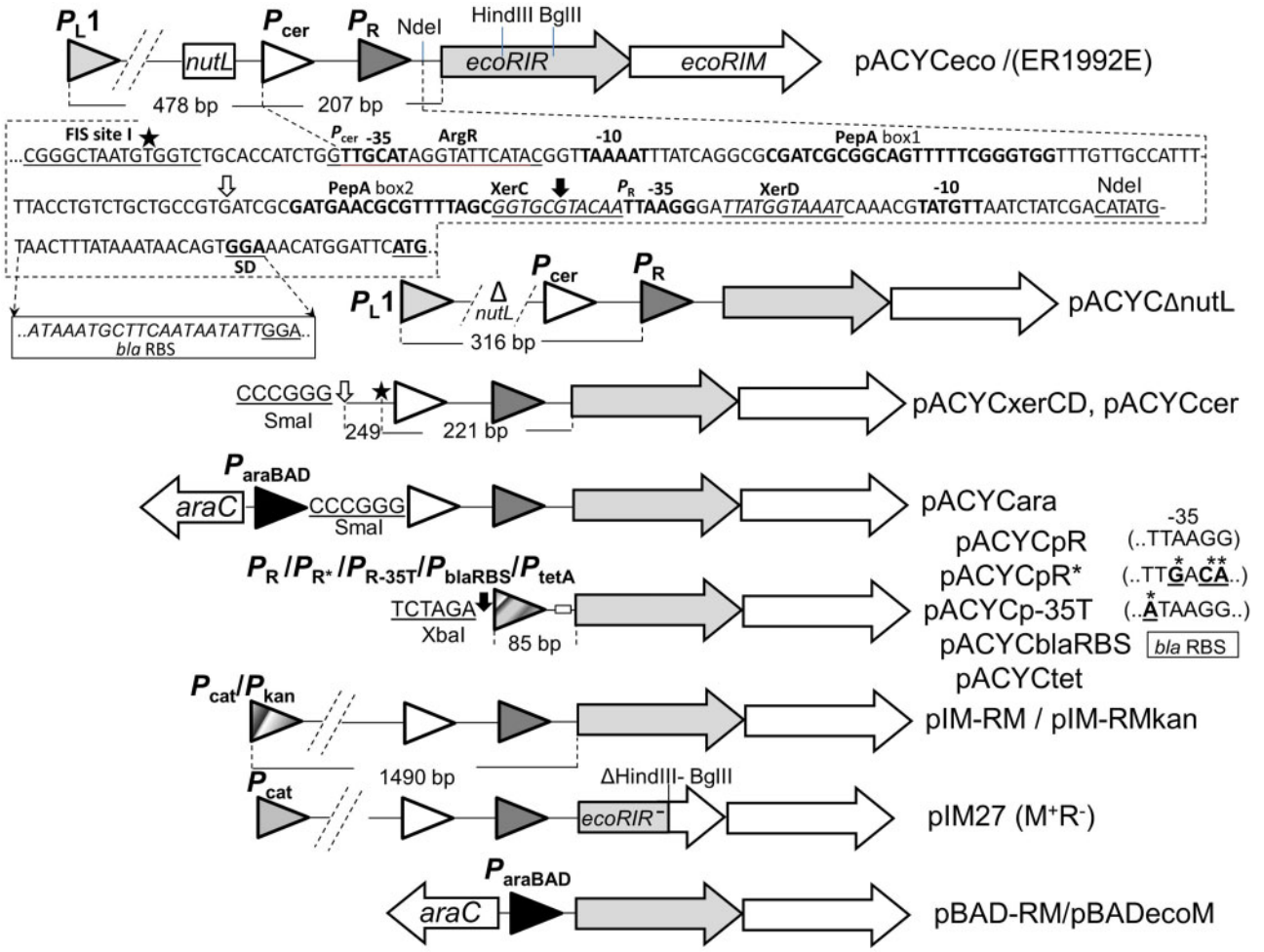

Figure 2. Scheme of key features of plasmid constructions used in this study. The set of plasmids bearing the EcoRI R–M system originating from pACYCeco or pIM-RM. Shown are important details in the sequence of the P_R promoter region structure, including its upstream vicinity that differed from each other. The names of plasmid constructs and modified promoters are given.

We reasoned that the only possible source of upstream transcription for the EcoRI operon in the pIM-RM plasmid was the constitutive promoter for the *cat* gene. Indeed, deletion of the *cat* promoter in pIM-RM or replacement with a DNA cassette carrying the kanamycin resistance gene (pIM-RMkan) restored a high phage restriction phenotype that was comparable to pACYCeco (Supplementary Table 3) by decreasing R–M expression (data not shown). A similar effect was obtained after transfer of *ecoRIRM* operon from pIM-RM to pBR322-derivative plasmid (data not shown).

3.3. A higher REase production was associated with R–M system instability and SOS response induction

Next, we tested whether high (pIM-RM) or low (pACYCeco) levels of *ecoRIR* gene expression differentially affected the bacterial SOS response. We examined the cells by microscopy and found high levels of filamentation, a typical result of the SOS response.[61,62] Filamentation was seen only for cells with the pIM-RM plasmid, but not for pACYCeco (Fig. 5A). To measure this phenomenon quantitatively, we used SOS-*gfp/yfp* reporter strains and found the levels of fluorescence to be high for cells carrying pIM-RM (Fig. 5B). *Escherichia coli* with pACYCeco or its derivatives, pACYCp and pACYCcer, did not show a similar effect (data not shown). These results indicated that the autorestriction of host DNA resulting from elevated cellular REase concentrations likely contributed to substantial SOS induction. We further measured the impact of REase regulation on cell viability by mixing an equal number of cells; an isogenic R-deficient strain (no plasmid) with the R-proficient strains (pACYCeco or pIM-RM), and then performed long-term competition assays. The mixed cultures were grown in rich media without antibiotic selection, and cell ratios were measured immediately at the start of growth and then again after ~60 generations. The strain carrying pIM-RM showed a significant decrease in cell viability when mixed with the restriction deficient control, when compared with the relatively balanced growth stability exhibited by the pACYCeco and plasmid (pIM27 R^-M^+) control strains (Fig. 5C).

3.4. The higher the R–M system expression, the less efficient phage DNA restriction

To determine whether the high levels of *ecoRIRM* expression resulted in more effective methylation of invading DNA, we supplied cells carrying pACYCeco or pIM-RM with an arabinose inducible MTase (pBAD-ecoM). Upon induction of the MTase, we observed a significant reduction of restriction in pACYCeco-carrying cells (Fig. 6A) and a loss of the permanent SOS-induction in pIM-RM

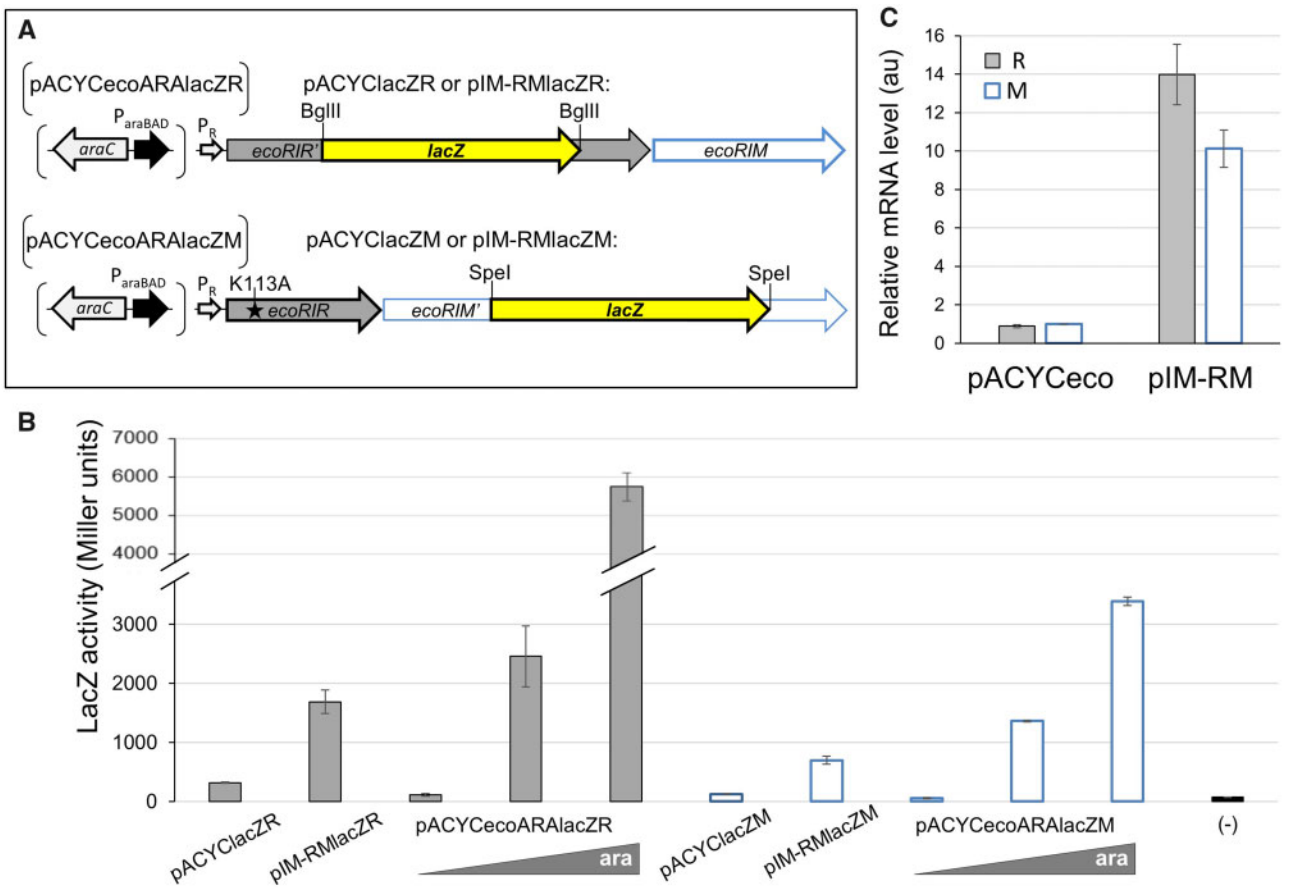

Figure 3. The EcoRI R–M expression level generated by pACYCeco and pIM-RM plasmid constructs. The *LacZ* reporter gene was used to construct translational fusions with *ecoRIR* endonuclease (pACYClacZR, pIM-RMLacZR, and pACYCecoARAlacZR) and *ecoRIM* methyltransferase (pACYClacZM, pIM-RMLacZM, and pACYCecoARAlacZM) genes. (A) Schematic diagram of plasmid constructs. Additional fragment carrying the inducible arabinose promoter upstream of P_R is shown in brackets as opposed to P_R constitutive expression. (B) The relative expression of *lacZ* measured in the Miller test, which reflected the levels of REase and MTase productions from tested plasmids. Note that in the case of bacteria harboring inducible pACYCecoARAlacZR or pACYCecoARAlacZM plasmids, 2-h induction of L-arabinose was applied in 0 (0.2% glucose), 0.04% and 0.4% concentrations, respectively. (C) The relative level of *ecoRIR* and *ecoRIM* gene transcripts tested by RT-qPCR.

carrying cells (Fig. 6B and C). We confirmed that the high *ecoRIRM* expression level was primarily linked with an overmethylation of foreign DNA and with the loss of the phage restriction phenotype. The results from these experiments with the EcoRI R–M system helped to explain the observation that higher R–M gene expression correlated with lower relative restriction of phage DNA and vice versa. It appears then that bacteriophage resistance may only be potent when methylation and restriction are balanced and their gene expression is sustained at very low levels.

3.5. The Xer/*cer* recombination system embedded in the P_R promoter region was dispensable for R–M system expression

The genetic elements comprising the Xer/*cer* recombination system overlapped the P_R promoter region (Fig. 2), so it is reasonable to assume that this region might contribute to the repression of the *ecoRIRM* operon. Previous reports have demonstrated that the synaptic complex formed during plasmid dimer resolution can block RNA polymerase from accessing the P_R promoter, and that the P_R region may be occupied by XerCD recombinases and the FIS protein during P_{cer} repression in plasmid monomers.[54] To test whether binding sites for Xer/*cer* proteins were embedded in the EcoRI main promoter, P_R (Fig. 2), impact expression, we used our phage restriction assay in bacterial strains with defective XerC, XerD, ArgR, and PepA.[63] The results showed no significant change in the DNA restriction properties for cells containing pACYCeco (Supplementary Fig. S3). We also removed the P_{cer} promoter along with Xer/*cer*-binding sites for recombination and accessory proteins (plasmids: pACYCcer, pACYCxerCD, and pACYCpR) (Supplementary Table 1). We observed only a slight increase in REase production that still allowed for a high level of antiviral functioning (Fig. 7; Supplementary Table S3).

To further characterize the minimum promoter required by the EcoRI R–M operon to maintain high levels of phage restriction, we constructed several pACYCeco derivatives, where the P_R promoter was shortened[36] or certain elements were replaced (Fig. 7). We did not see an increase in DNA restriction levels even, when the -35 box of the main P_R promoter was substituted with a consensus promoter sequence (TTAAGG→TTGACA; pACYCp*) (Fig. 7). There was a significant loss in restriction activity with a single nucleotide change (TTAAGG→ATAAGG; pACYC-35T) to the -35 hexamer of P_R (Fig. 7). This might have suggested that P_R could not function as a simple -10 extended promoter,[64,65] despite the structural potential

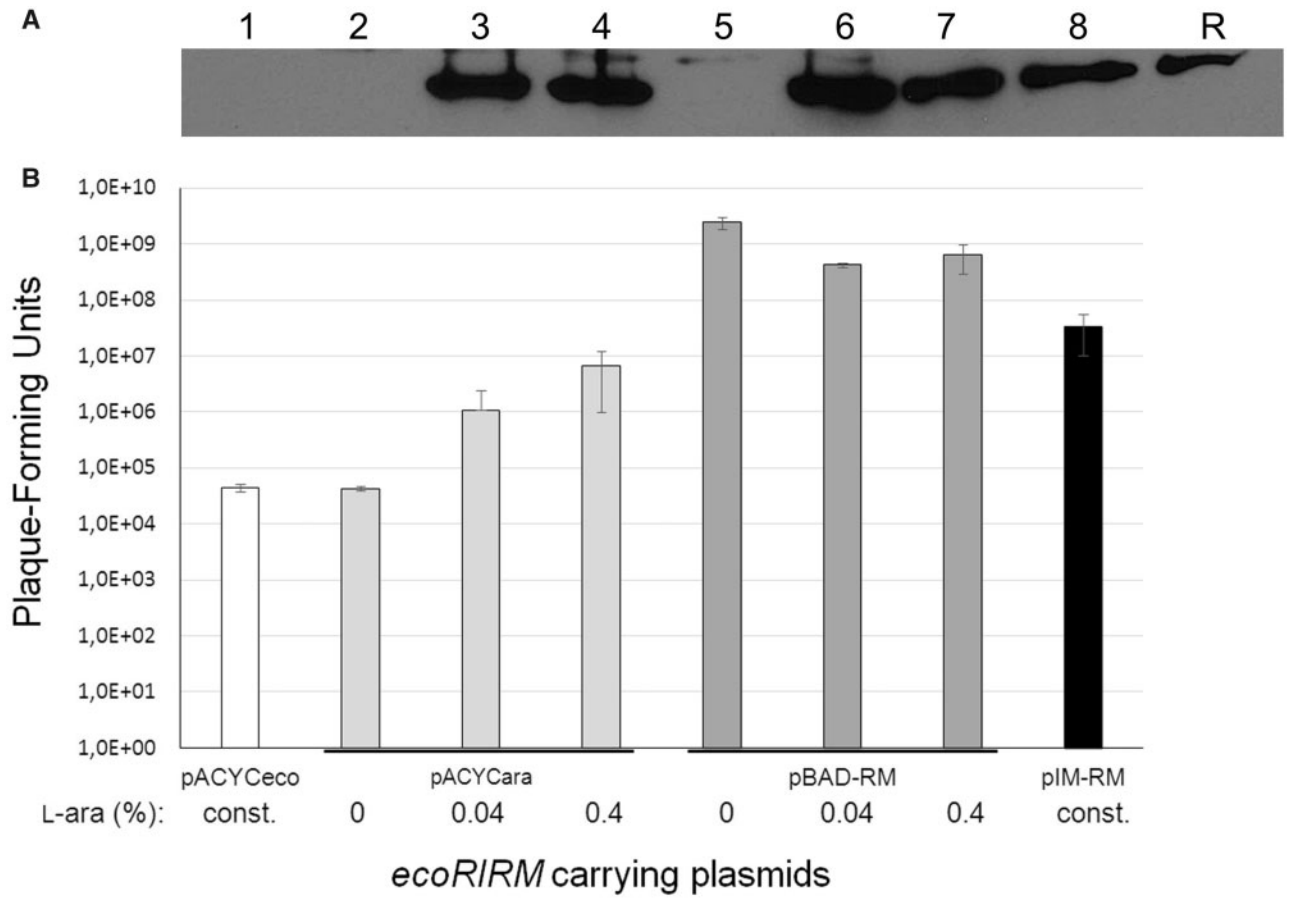

Figure 4. Phage restriction is inversely proportional to the observed EcoRI R–M expression level. (A) Western blot analysis of the levels of EcoRI endonuclease production in bacterial crude extracts from the different modifying promoter region, after constitution [Lane 1, pACYCeco; Lane 2, pACYCara (P_{araBAD} under repression with 0.2% glucose); Lane 8, pIM-RM] and inducing expression of R–M genes [Lanes 3 and 4, pACYCara after 2 h 0.04% and 0.4% L-arabinose (L-ara) induction, respectively; Lanes 6 and 7, pBAD-RM after 2 h 0.04% and 0.4% L-ara induction, respectively; Lane 5, pBAD-RM (P_{araBAD} under repression with 0.2% glucose)]; and Lane R, the R.EcoRI protein preparation. ER1992 cells were grown to the late exponential phase. The nitrocellulose membrane was probed with rabbit polyclonal anti-R.EcoRI antiserum. ECL chemiluminescence with horseradish peroxidase-coupled goat anti-rabbit IgG was used to visualize the products. Culture samples were normalized by OD_{600}. (B) PFUs of the previously mentioned plasmid-bearing bacteria.

(TGTTAATTC).[36] In another construct, we inserted the EcoRI wild-type (WT) core promoter elements (-35 and -10 sequences) into the weak promoter of the tetracycline resistance gene (P_{tetA}). The construct maintained the WT leader sequence for the transcript (pACYCtet) (Figs. 2 and 7; Supplementary Table S3). Cells with this plasmid showed a dramatic loss in phage restriction (Supplementary Table S3). The untranslated upstream element for the promoter from *bla* is recognized as important in expression and is in some cases indispensable to maintaining the fine-tuning in gene expression.[66,67] Leaving the WT P_R promoter intact, we then replaced the region downstream of -10 hexamer with one similar to that from the *bla* gene promoter to create pACYCblaRBS (Fig. 2). The construct used part of the native RBS from *bla*, and cells bearing it exhibited low levels (comparable to pACYCtet) of λ DNA restriction in the phage assay (Fig. 7). Overall, these results indicated a very high degree of optimization for each element present in the WT EcoRI R–M promoter region.

4. Discussion

In general, studies focused on dissecting the detailed mechanisms involved in the regulation of gene expression tend to dedicate more attention to operon coding sequences rather than the transcriptional signals coming from the sequences outside the protein-coding regions. In the present report, we used the EcoRI R–M system as a model to focus on how operon adjacent sequences affected the balance between the REase and MTase levels in Type II R–M systems.

4.1. Sequences flanking the *ecoRIRM* operon affect the expression of the REase and MTase levels

We tested the possibility that the ratio of restriction and modification activities must operate within a narrow window of variation in order to balance protection from invading DNA and unwanted cell death from autorestriction, in a similar manner to the activity of a toxin/antitoxin systems.[9] The results from our plasmid-based regulatory studies suggested that the activity and function of an R–M system as well as its impact on cellular fitness were linked to the genotype of the host bacterium. Our finding that the cellular levels and ratios between the REase and MTase could be different in different genetic contexts was in agreement with the results from other studies of the EcoRI operon.[10]

ColE1-like plasmids are small, high copy and mobilizable replicons, which are perceived as the main vectors for the acquisition,

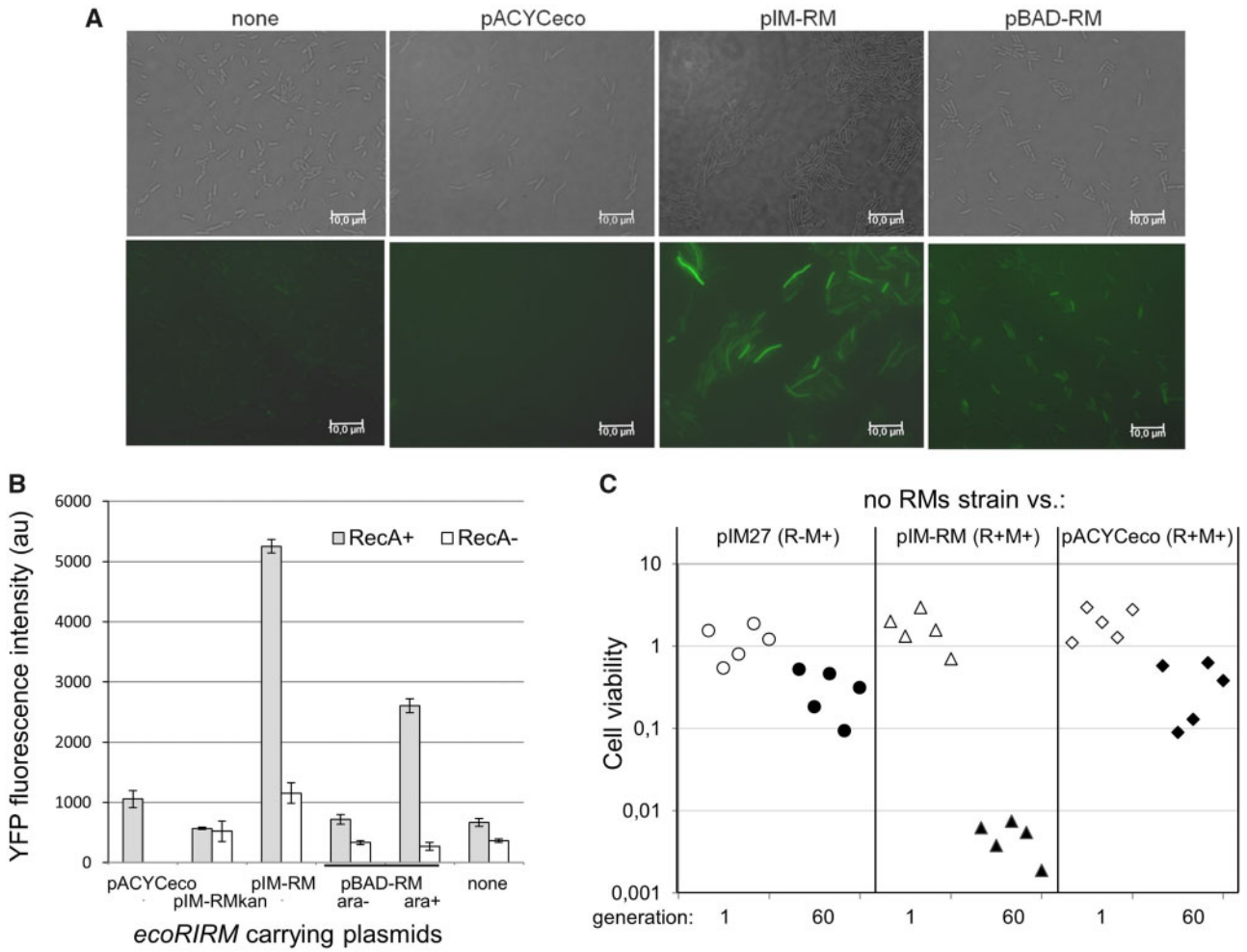

Figure 5. The SOS response induction and R–M system instability in cells carrying the plasmid with the high R–M genes expression. (A) *Escherichia coli* strain SS996, where the *gfp* gene is under the control of the P_{sulA} promoter, was transformed with the following plasmids: pIM-RM (high R–M genes expression), pACYCeco (low R–M genes expression) and pBAD-RM, where REase expression was induced by 0.04% of L-arabinose for 2 h. Light and fluorescence microscopy images were captured. (B) The SOS response was also measured quantitatively for the same plasmids in *E. coli* strains with the YFP reporter gene under the control of the P_{sulA} promoter in the background of *recA*$^+$ (MP060) and *recA*$^-$ (MP064). As a control, the plasmid, pIM-RM, with a kanamycin resistance cassette inserted in *cat* gene upstream of the P_R promoter for REase gene (pIM-RMkan) was used, as well as strains without plasmids. (C) Plasmids pIM-RM and pACYCeco (both restriction proficient R^+M^+; high and low REase producers) were challenged using a 3-day growth competition assay to generate the co-cultures with the MG1655 strain without a plasmid (no RM system). As a control, plasmid with a restriction-negative variant was also used (pIM27: R^-M^+). Briefly, strains without plasmid were mixed 1:1 with cells carrying plasmid with the R–M system variant and allowed to grow in the co-cultures for 60 generations. Chloramphenicol resistance was used as a selection marker for CFUs of cells carrying the R–M system variants. The cell viability was measured at Generations 1 and 60, and calculated as the ratio of the CFUs on LB agar supplemented with chloramphenicol to the CFUs obtained on LB agar.

evolution, and dissemination of a wide variety of genes including the R–M determinants.[68–70] Our database survey revealed the existence of several homologs of the EcoRI R–M system located on ColE1-like plasmids from the *Enterobacteriaceae* family (*E. coli*, GenBank: ANUR01000054.1, CYBB01000052.1, J01675.1, NZ_CCRA01000002.1, and NZ_LDDU01000010.1; *Shigella sonnei*, GenBank: M97479; and *Citrobacter youngae*, GenBank: ABWL02000046.1). Notably, in all these cases, the sequences within the promoters and the upstream regions were almost identical. The location of the crossover site between the two Xer-binding sites in the *cer* region, which affects the 3′ portion of the -35 sequence,[43] may indicate that this promoter region is recruited randomly after each recombination event when a hybrid DNA sequence is formed from two different widespread *cer*-like sites.[41,71] The XerC/D-mediated generation of co-integrates between different plasmids increased the possibilities of their rearrangements and further horizontal dissemination among Gram-negative rods.[70,72] A good example of this possibility was the P6 plasmid carrying *ecoRIRM* genes in *S. sonnei* (GenBank M9479). The loss of a single T in the sequence considered to be the -35 box of P_R (TTAAGG→ATAAGG)[36] may be the result of recombinational crossover at this locus.[40,42,43] Other experiments have already shown that *cer* hybrids may lose all conserved and essential nucleotides at the -35 box consensus positions.[43] Given these insights, we think that one important factor for efficient control of *ecoRIRM* expression is the -10 box of the promoter because it can act as a classical extended -10 promoter.[36,64,65] For the constructs and conditions tested, we showed that the extended -10 box could not compensate in the cases of a poor or absent -35 sequence (Fig. 7). These fluctuations in the R–M system promoter strength may be crucial to the predator/prey roles in the restriction/host relationship and to the adaptive impacts on horizontal

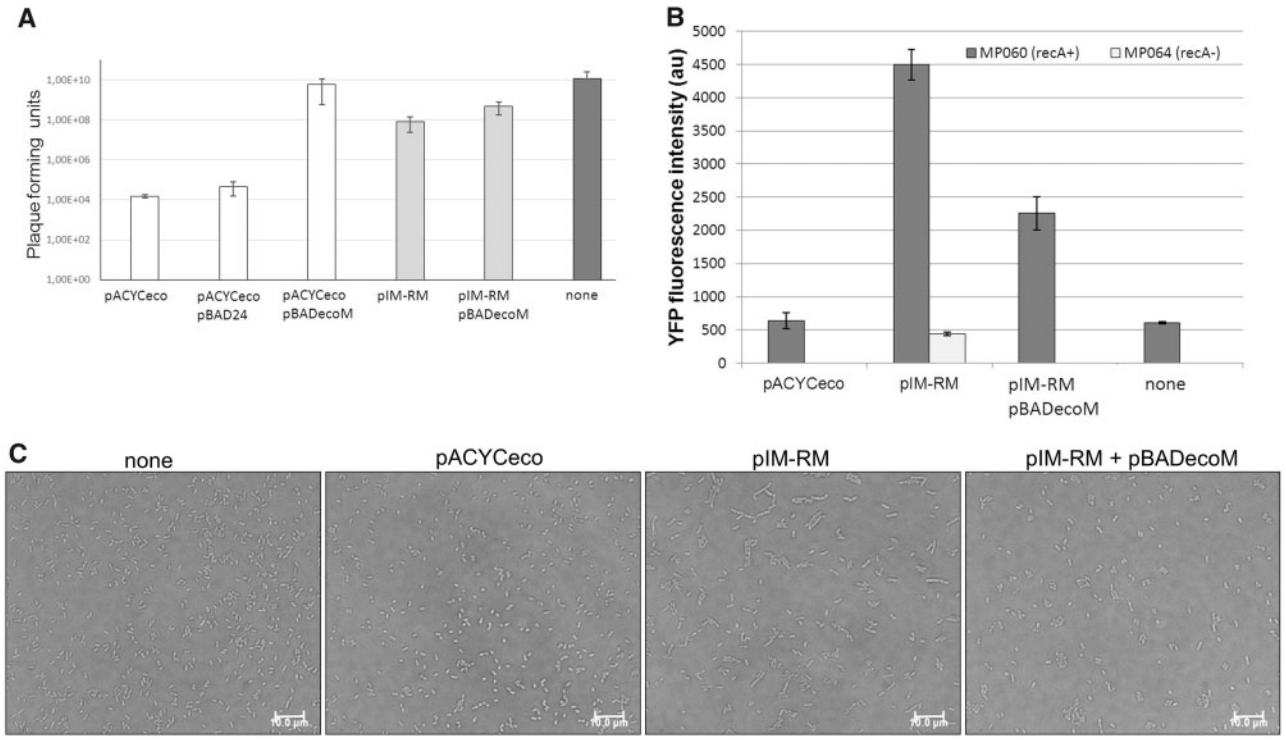

Figure 6. Overmethylation alleviates the highly restrictive phenotype and suppresses the autorestriction defects. *Escherichia coli* ER1992 (A) or MP060 cells (B, C) carrying pACYCeco or pIM-RM plasmids were co-transformed with pBADecoM carrying the arabinose inducible *ecoRIM* gene. MTase expression was induced with 0.04% for 1 h at 37 °C (A) or overnight (B, C). Then, a quantitative assay for restriction λ_{vir} phage DNA was performed (A), and SOS-inducing YFP fluorescence was measured (B) or microscopy of cells was conducted (C).

gene transfer at the stage of gene establishment and maintenance of a new host.

4.2. A high expression level of R–M results in loss of cell viability

R–M system expression imbalance can result in a loss of viability and cell death or may even give rise to restriction-deficient phenotypes in some hosts.[14,29,30,73–79] Examples of these phenomena include the accumulation of single-strand breaks at non-cognate sites observed when EcoRV REase is overproduced,[11] and the autorestriction-based reduction in cell fitness that occurs after the induction of R–M expression leading to prominent rearrangements that inactivate the *mboIIR* REase gene by IS elements.[80] Furthermore, long-term mixed culture experiments suggest strong selective pressure against the presence of a DNA cutting agent that affects cell fitness.[81] To the best of our knowledge, most experiments related to the contribution of R–M systems to plasmid stability were performed in a single host strain making it difficult to directly estimate the impact of the post-segregational host killing phenomenon in a broader context,[82,83] e.g. as an important factor, which could play a role in the competition between populations of strains with different restriction phenotypes. In addition, the growth conditions may significantly affect the CoE1 plasmid copy, so it should also be considered as a dosage-dependent gene regulation factor,[84,85] as well as the presence of the growth spatial structure (as in solid medium), which may favor the cells with a suicide strategy.[86,87]

Death of cells triggered by the degradation of their own DNA after R–M gene complex loss is linked to an enhanced decrease in methylated sites and exposure to cognate REase action.[31,33] We used SOS response-reporter strains as a direct approach to understanding the impacts of optimal and sub-optimal levels in R–M enzyme activities. Cells carrying a plasmid with higher R–M enzyme production (pIM-RM) exhibited cell filamentation and a typical SOS response phenotype (Fig. 5A and B). It was reported that accidental DNA autorestriction events occur even in the balanced EcoRI R–M system.[88] It was also reported that atypical cells with unusually high accumulations of Esp1396I R–M enzymes undergo filamentation and lysis.[12] Therefore, it is not surprising that we could observe substantial filamentation with an overexpressed R–M system, where the cognate protection was insufficient. A high REase to DNA target site ratio can generate the undesirable cuts at non-protected and/or non-specific sites (star sites),[78,89] which is a typical feature for REases conferring promiscuity toward the target site, such as the extensively studied EcoRI REase.[90,91] Because the pIM-RM bearing strain was suppressed from autorestriction by the overmethylation of EcoRI MTase (Fig. 6B and C), the cleavage specificity of the EcoRI REase was unaltered at higher levels. In parallel, the restriction ability displayed by the pACYCeco-carrying strain decreased dramatically under the same conditions (Fig. 6A). We also used growth competition assays under non-selective conditions in recombination-proficient strains as an indirect measure of the effects of R–M system regulation. In long-term co-cultures, the isogenic strain without R–M system genes was competing against an R–M proficient strain (high or low producer). The higher R–M producer (pIM-RM) failed to compete with the restriction deficient strain (Fig. 5C). Notably, the pIM-RM culture colonies that formed on agar plates were different in size, and the smallest was composed of mostly filamentous cells. In

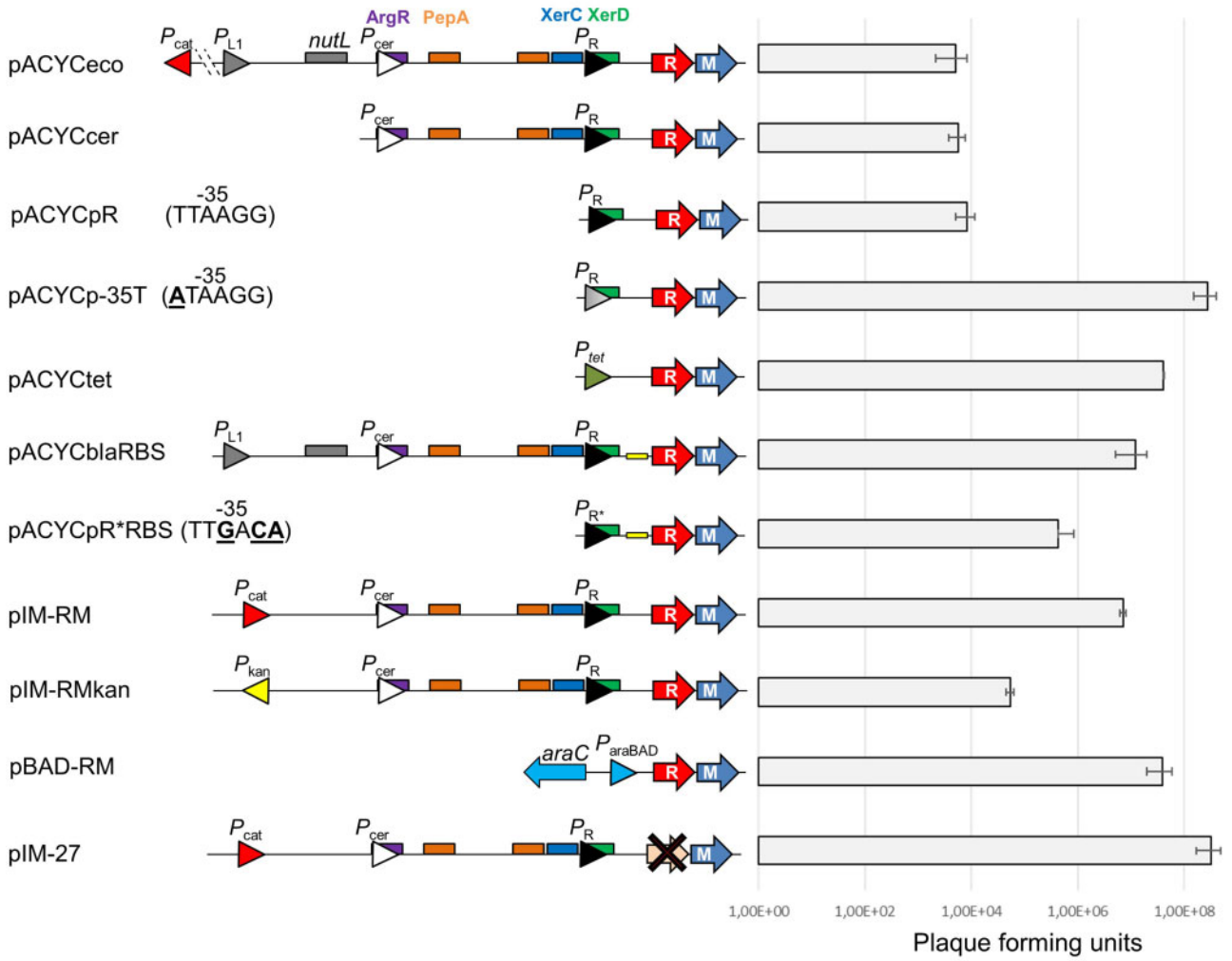

Figure 7. Strength of the promoter region governs the efficiency of relative restriction. (A) Western blot analysis of the levels of EcoRI REase and MTase production in bacterial crude extracts, after constitutive R–M expression from different modifying promoter regions from late exponential grown ER1992 cells (Lanes 1–9), with the exception of pBAD-RM, and was subjected to 2-h induction by 0.04% L-arabinose (Lane 10). In the last case, 10-fold less pellet was applied. The nitrocellulose membrane was probed simultaneously with rabbit polyclonal anti-R.EcoRI and anti-M.EcoRI antisera, and ECL chemiluminescence with horseradish peroxidase-coupled goat anti-rabbit IgG was used to visualize the products. Culture samples were normalized by OD_{600}. *The location of the unknown antigen protein, confirmed that equivalent amounts of proteins were loaded on each lane. (B) Relative restriction of the above constructs expressed as the efficiency of plating.

contrast, the lower R–M producer (pACYCeco) was not outcompeted by the restriction-deficient strain. The observed fitness defect of cells carrying the high level R–M enzyme producer may reflect the need for longer-term adaptation of the R–M system in order to match the genetic background and the molecular interrelationship network of the new host.[92] It may also support the observation that chromosomal R–M systems are better adapted to fulfill their biological function. Indeed, the majority of all discovered R–M systems are chromosomal and rarely present on low-copy replicons.[93,94] However, it has recently been shown that the strength of post-segregational host killing is not simply a function of the number of recognition sites present on the chromosome but also a reflection of the properties of a particular R–M system, including protein stability.[17,88,95,96]

5. Conclusion

We think that the balance in restriction and modification activities from a single-copy R–M system is operating very close to the edge of imbalance, and this has profound biological implications considering the role of the REase in efficiently degrading foreign DNA before the methylation arm of the system renders the DNA resistant. Alterations in gene copy number or promoter strength that affect R–M gene expression and enzyme concentration may be triggered by specific nutritional and/or growth conditions, as well as by the impact of the new genetic context resulting from rearrangement of R–M genes into a new host. Regulation of gene expression for chromosomal R–M systems appears to be mostly free of these external (environmental) factors.

Acknowledgments

We thank Prof. Ichizo Kobayashi (University of Tokyo) for the gift of the antibodies against R and M of *Eco*RI RM system. We acknowledge also Dr Robert Lintner for his great help at the stage of manuscript editing.

Supplementary data

Supplementary data are available at *DNARES* online.

Funding

This work was supported by Faculty of Biology of University of Gdansk (Grant No. 538-L135-B548-17 to K.W.).

Conflict of interest

None declared.

References

1. Labrie, S.J., Samson, J.E. and Moineau, S. 2010, Bacteriophage resistance mechanisms, *Nat. Rev. Microbiol.*, **8**, 317–27.
2. Tock, M.R. and Dryden, D.T. 2005, The biology of restriction and anti-restriction, *Curr. Opin. Microbiol.*, **8**, 466–72.
3. Makarova, K.S., Wolf, Y.I. and Koonin, E.V. 2013, Comparative genomics of defense systems in archaea and bacteria, *Nucleic Acids Res.*, **41**, 4360–77.
4. Vasu, K. and Nagaraja, V. 2013, Diverse functions of restriction-modification systems in addition to cellular defense, *Microbiol. Mol. Biol. Rev.*, **77**, 53–72.
5. Doron, S., Melamed, S., Ofir, G., et al. 2018, Systematic discovery of antiphage defense systems in the microbial pangenome, *Science*, **359**, eaar4120.
6. Blakely, G. and Murray, N. 2006, Control of the endonuclease activity of type I restriction-modification systems is required to maintain chromosome integrity following homologous recombination, *Mol. Microbiol.*, **60**, 883–93.
7. Surby, M. and Reich, N. 1996, Contribution of facilitated diffusion and processive catalysis to enzyme efficiency: implications for the EcoRI restriction-modification system, *Biochemistry*, **35**, 2201–8.
8. Surby, M. and Reich, N. 1996, Facilitated diffusion of the EcoRI DNA methyltransferase is described by a novel mechanism, *Biochemistry*, **35**, 2209–17.
9. Mruk, I. and Kobayashi, I. 2014, To be or not to be: regulation of restriction-modification systems and other toxin-antitoxin systems, *Nucleic Acids Res.*, **42**, 70–86.
10. Ichige, A. and Kobayashi, I. 2005, Stability of EcoRI restriction-modification enzymes in vivo differentiates the EcoRI restriction-modification system from other postsegregational cell killing systems, *J. Bacteriol.*, **187**, 6612–21.
11. Taylor, J., Goodall, A., Vermote, C. and Halford, S. 1990, Fidelity of DNA recognition by the EcoRV restriction/modification system in vivo, *Biochemistry*, **29**, 10727–33.
12. Morozova, N., Sabantsev, A., Bogdanova, E., et al. 2016, Temporal dynamics of methyltransferase and restriction endonuclease accumulation in individual cells after introducing a restriction-modification system, *Nucleic Acids Res.*, **44**, 790–800.
13. Kong, H., Lin, L.F., Porter, N., et al. 2000, Functional analysis of putative restriction-modification system genes in the *Helicobacter pylori* J99 genome, *Nucleic Acids Res.*, **28**, 3216–23.
14. Katna, A., Boratynski, R., Furmanek-Blaszk, B., Zolcinska, N. and Sektas, M. 2010, Unbalanced restriction impairs SOS-induced DNA repair effects, *J. Microbiol. Biotechnol.*, **20**, 30–8.
15. Handa, N., Ichige, A., Kusano, K. and Kobayashi, I. 2000, Cellular responses to postsegregational killing by restriction-modification genes, *J. Bacteriol.*, **182**, 2218–29.
16. Handa, N. and Kobayashi, I. 1999, Post-segregational killing by restriction modification gene complexes: observations of individual cell deaths, *Biochimie*, **81**, 931–8.
17. Nagamalleswari, E., Rao, S., Vasu, K. and Nagaraja, V. 2017, Restriction endonuclease triggered bacterial apoptosis as a mechanism for long time survival, *Nucleic Acids Res.*, **45**, 8423–34.
18. Aras, R., Takata, T., Ando, T., van der Ende, A. and Blaser, M. 2001, Regulation of the HpyII restriction-modification system of *Helicobacter pylori* by gene deletion and horizontal reconstitution, *Mol. Microbiol.*, **42**, 369–82.
19. Jeltsch, A. and Pingoud, A. 1996, Horizontal gene transfer contributes to the wide distribution and evolution of type II restriction-modification systems, *J. Mol. Evol.*, **42**, 91–6.
20. Nobusato, A., Uchiyama, I., Ohashi, S. and Kobayashi, I. 2000, Insertion with long target duplication: a mechanism for gene mobility suggested from comparison of two related bacterial genomes, *Gene*, **259**, 99–108.
21. Naderer, M., Brust, J., Knowle, D. and Blumenthal, R. 2002, Mobility of a restriction-modification system revealed by its genetic contexts in three hosts, *J. Bacteriol.*, **184**, 2411–9.
22. Oliveira, P.H., Touchon, M. and Rocha, E.P. 2016, Regulation of genetic flux between bacteria by restriction-modification systems, *Proc. Natl Acad. Sci. U.S.A.*, **113**, 5658–63.
23. Donahue, J., Israel, D., Peek, R., Blaser, M. and Miller, G. 2000, Overcoming the restriction barrier to plasmid transformation of *Helicobacter pylori*, *Mol. Microbiol.*, **37**, 1066–74.
24. Debacker, O. and Colson, C. 1991, Transfer of the genes for StyLTI restriction-modification system of *Salmonella typhimurium* to strains lacking modification ability results in death of the recipient cells and degradation of their DNA, *J. Bacteriol.*, **173**, 1328–30.
25. Mruk, I. and Blumenthal, R.M. 2008, Real-time kinetics of restriction-modification gene expression after entry into a new host cell, *Nucleic Acids Res.*, **36**, 2581–93.
26. Roberts, R. and Halford, S. 1993, Type II restriction endonucleses. In: Linn, S., Lloyd, R. and Roberts, R. (eds), *Nucleases*, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, pp. 35–88.
27. Pingoud, A., Wilson, G.G. and Wende, W. 2016, Type II restriction endonucleases - a historical perspective and more, *Nucleic Acids Res.*, **44**, 8011.
28. Nakayama, Y. and Kobayashi, I. 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**, 6442–7.
29. Heitman, J., Zinder, N.D. and Model, P. 1989, Repair of the *Escherichia coli* chromosome after in vivo scission by the EcoRI endonuclease, *Proc. Natl Acad. Sci. U.S.A.*, **86**, 2281–5.
30. Heitman, J., Ivanenko, T. and Kiss, A. 2002, DNA nicks inflicted by restriction endonucleases are repaired by a RecA- and RecB-dependent pathway in *Escherichia coli*, *Mol. Microbiol.*, **33**, 1141–51.
31. Naito, T., Kusano, K. and Kobayashi, I. 1995, Selfish behavior of restriction-modification systems, *Science*, **267**, 897–9.
32. Naito, Y., Naito, T. and Kobayashi, I. 1998, Selfish restriction modification genes: resistance of a resident R/M plasmid to displacement by an incompatible plasmid mediated by host killing, *Biol. Chem.*, **379**, 429–36.
33. Kusano, K., Naito, T., Handa, N. and Kobayashi, I., , and 1995, Restriction-modification systems as genomic parasites in competition for specific sequences,*Proc Natl Acad Sci USA* **92**, 11095–9.
34. Greene, P., Gupta, M., Boyer, H., Brown, W. and Rosenberg, J. 1981, Sequence analysis of the DNA encoding the EcoRI endonuclease and methylase, *J. Biol. Chem.*, **256**, 2143–53.
35. Mruk, I., Liu, Y., Ge, L. and Kobayashi, I. 2011, Antisense RNA associated with biological regulation of a restriction-modification system, *Nucleic Acids Res.*, **39**, 5622–32.
36. Liu, Y. and Kobayashi, I. 2007, Negative regulation of the EcoRI restriction enzyme gene is associated with intragenic reverse promoters, *J. Bacteriol.*, **189**, 6928–35.
37. Liu, Y., Ichige, A. and Kobayashi, I. 2007, Regulation of the EcoRI restriction-modification system: identification of ecoRIM gene promoters

and their upstream negative regulators in the ecoRIR gene, *Gene*, **400**, 140–9.
38. Newman, A., Rubin, R., Kim, S. and Modrich, P. 1981, DNA sequences of structural genes for EcoRI DNA restriction and modification enzymes, *J. Biol. Chem.*, **256**, 2131–9.
39. Alen, C., Sherratt, D. and Colloms, S. 1997, Direct interaction of aminopeptidase A with recombination site DNA in Xer site-specific recombination, *EMBO J.*, **16**, 5188–97.
40. Zakova, N. and Szatmari, G. 1995, Site-specific recombination between ColE1 cer and NTP16 nmr sites in vivo, *Mol. Gen. Genet.*, **247**, 509–14.
41. Summers, D., Yaish, S., Archer, J. and Sherratt, D. 1985, Multimer resolution systems of ColE1 and ColK: localisation of the crossover site, *Mol. Gen. Genet.*, **201**, 334–8.
42. Summers, D. and Sherratt, D. 1988, Resolution of ColE1 dimers requires a DNA-sequence implicated in the 3-dimentional organization of the cer site, *EMBO J.*, **7**, 851–8.
43. Summers, D. 1989, Derivatives of ColE1 cer show altered topological specificity in site-specific recombination, *EMBO J.*, **8**, 309–15.
44. Betlach, M., Hershfield, V., Chow, L., Brown, W., Goodman, H. and Boyer, H. 1976, Restriction endonuclease analysis of the bacterial plasmid controlling the EcoRI restriction and modification of DNA, *Fed. Proc.*, **35**, 2037–43.
45. Miller, C. and Cohen, S. 1978, Phenotypically cryptic EcoRI endonuclease activity specified by the ColE1 plasmid, *Proc. Natl Acad. Sci. U.S.A.*, **75**, 1265–9.
46. Yoshimori, R., Roulland, D. and Boyer, H. 1972, R factor-controlled restriction and modification of deoxyribonucleic acid: restriction mutants, *J. Bacteriol.*, **112**, 1275–9.
47. Smith, H., Humphreys, G., Willshaw, G. and Anderson, E. 1976, Characterization of plasmid coding for the restriction endonuclease EcoRI, *Mol. Gen. Genet.*, **143**, 319–25.
48. Chang, S. and Cohen, S. 1977, In vivo site-specific genetic recombination promoted by the EcoRI restriction endonuclease, *Proc. Natl Acad. Sci. U.S.A.*, **74**, 4811–5.
49. Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989, *Molecular Cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.
50. Kellenberger, G., Zichichi, M. and Weigle, J. 1960, Mutations affecting the density of bacteriophage, *Nature*, **187**, 161–2.
51. Huggett, J., Dheda, K., Bustin, S. and Zumla, A. 2005, Real-time RT-PCR normalisation; strategies and considerations, *Genes Immun.*, **6**, 279–84.
52. Pfaffl, M.W. 2001, A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acids Res.*, **29**, e45.
53. Cheng, S., Kim, R., King, K., Kim, S. and Modrich, P. 1984, Isolation of gram quantities of EcoRI restriction and modification enzymes from an overproducing strain, *J. Biol. Chem.*, **259**, 1571–5.
54. Blaby, I. and Summers, D. 2009, The role of FIS in the Rcd checkpoint and stable maintenance of plasmid ColE1, *Microbiology*, **155**, 2676–82.
55. Guhathakurta, A. and Summers, D. 1995, Involvement of ArgR and PepA in the pairing of ColE1 dimer resolution sites, *Microbiology*, **141**, 1163–71.
56. Stirling, C., Szatmari, G., Stewart, G., Smith, M. and Sherratt, D. 1988, The arginine repressor is essential for plasmid-stabilizing site-specific recombination at the ColE1 cer locus, *EMBO J.*, **7**, 4389–95.
57. Giladi, H., Murakami, K., Ishihama, A. and Oppenheim, A. 1996, Identification of an UP element within the IHF binding site at the P(L)1-P(L)2 tandem promoter of bacteriophage lambda, *J. Mol. Biol.*, **260**, 484–91.
58. Parks, A., Court, C., Lubkowska, L., Jin, D., Kashlev, M. and Court, D. 2014, Bacteriophage lambda N protein inhibits transcription slippage by *Escherichia coli* RNA polymerase, *Nucleic Acids Res.*, **42**, 5823–9.
59. Wilson, H., Yu, D., Peters, H., Zhou, J. and Court, D. 2002, The global regulator RNase III modulates translation repression by the transcription elongation factor N, *EMBO J.*, **21**, 4154–61.
60. Grabowski, G., Jeltsch, A., Wolfes, H., Maass, G. and Alves, J. 1995, Site-directed mutagenesis in the catalytic center of the restriction-endonuclease EcoRI, *Gene*, **157**, 113–8.
61. D'Ari, R. and Huisman, O. 1983, Novel mechanism of cell division inhibition associated with the SOS response in *Escherichia coli*, *J. Bacteriol.*, **156**, 243–50.
62. Drapeau, G.R., Gariépy, F. and Boulé, M. 1984, Regulation and SOS induction of division inhibition in *Escherichia coli* K12, *Mol. Gen. Genet.*, **193**, 453–8.
63. Colloms, S.D. 2013, The topology of plasmid-monomerizing Xer site-specific recombination, *Biochem. Soc. Trans.*, **41**, 589–94.
64. Graña, D., Youderian, P. and Susskind, M.M. 1985, Mutations that improve the ant promoter of *Salmonella* phage P22, *Genetics*, **110**, 1–16.
65. Keilty, S. and Rosenberg, M. 1987, Constitutive function of a positively regulated promoter reveals new sequences essential for activity, *J. Biol. Chem.*, **262**, 6389–95.
66. De Mey, M., Maertens, J., Boogmans, S., et al. 2010, Promoter knock-in: a novel rational method for the fine tuning of genes, *BMC Biotechnol.*, **10**, 26.
67. Kanhere, A. and Bansal, M. 2005, Structural properties of promoters: similarities and differences between prokaryotes and eukaryotes, *Nucleic Acids Res.*, **33**, 3165–75.
68. Mruk, I., Sektas, M. and Kaczorowski, T. 2001, Characterization of pEC156, a ColE1-type plasmid from *Escherichia coli* E1585-68 that carries genes of the EcoVIII restriction-modification system, *Plasmid*, **46**, 128–39.
69. Werbowy, O. and Kaczorowski, T. 2016, Plasmid pEC156, a naturally occurring *Escherichia coli* genetic element that carries genes of the EcoVIII restriction-modification system, is mobilizable among enterobacteria, *PLoS One*, **11**, e0148355.
70. Ares-Arroyo, M., Bernabe-Balas, C., Santos-Lopez, A., et al. 2018, PCR-based analysis of ColeE1 plasmids in clinical isolates and metagenomic samples reveals their importance as gene capture platforms, *Front. Microbiol.*, **9**, 469.
71. Sirois, S. and Szatmari, G. 1995, Detection of XerC and XerD recombinases in Gram-negative bacteria of the family Enterobacteriaceae, *J. Bacteriol.*, **177**, 4183–6.
72. Cameranesi, M.M., Morán-Barrio, J., Limansky, A.S., Repizo, G.D. and Viale, A.M. 2018, Site-specific recombination at XerC/D sites mediates the formation and resolution of plasmid co-integrates carrying a *bla*OXA-58-and Tn*aphA6*-resistance module in *Acinetobacter baumannii*, *Front. Microbiol.*, **9**, 66.
73. Brooks, J.E., Benner, J.S., Heiter, D.F., et al. 1989, Cloning the BamHI restriction modification system, *Nucleic Acids Res.*, **17**, 979–97.
74. Rice, M.R., Koons, M.D. and Blumenthal, R.M. 1999, Substrate recognition by the Pvu II endonuclease: binding and cleavage of CAG5mCTG sites, *Nucleic Acids Res.*, **27**, 1032–8.
75. Toksoy, E., Onsan, Z.I. and Kirdar, B. 2002, High-level production of TaqI restriction endonuclease by three different expression systems in *Escherichia coli* cells using the T7 phage promoter, *Appl. Microbiol. Biotechnol.*, **59**, 239–45.
76. Vipond, I.B. and Halford, S.E. 1996, Random mutagenesis targeted to the active site of the EcoRV restriction endonuclease, *Biochemistry*, **35**, 1701–11.
77. Hubácek, J., Holubová, I. and Weiserová, M. 1998, The effect of recA mutation on the expression of EcoKI and EcoR124I hsd genes cloned in a multicopy plasmid, *Folia Microbiol.*, **43**, 353–9.
78. Smith, M.D., Longo, M., Gerard, G.F. and Chatterjee, D.K. 1992, Cloning and characterization of genes for the PvuI restriction and modification system, *Nucleic Acids Res.*, **20**, 5743–7.
79. Mruk, I., Kaczorowski, T. and Witczak, A. 2019, Natural tuning of restriction endonuclease synthesis by cluster of rare arginine codons, *Sci. Rep.*, **9**, 5808.
80. Furmanek-Blaszk, B., Boratynski, R., Zolcinska, N. and Sektas, M. 2009, M1.MboII and M2.MboII type IIS methyltransferases: different specificities, the same target, *Microbiology*, **155**, 1111–21.

81. Rezulak, M., Borsuk, I. and Mruk, I. 2016, Natural C-independent expression of restriction endonuclease in a C protein-associated restriction-modification system, *Nucleic Acids Res*., **44**, 2646–60.
82. Kusano, K., Naito, T., Handa, N. and Kobayashi, I. 1995, Restriction-modification systems as genomic parasites in competition for specific sequence, *Proc. Natl Acad. Sci. U.S.A*., **92**, 11095–9.
83. Kulakauskas, S., Lubys, A. and Ehrlich, S.D. 1995, DNA restriction-modification systems mediate plasmid maintenance, *J. Bacteriol*., **177**, 3451–4.
84. Wegrzyn, G. 1999, Replication of plasmids during bacterial response to amino acid starvation, *Plasmid*, **41**, 1–16.
85. Williams, K., Savageau, M.A. and Blumenthal, R.M. 2013, A bistable hysteretic switch in an activator-repressor regulated restriction-modification system, *Nucleic Acids Res*., **41**, 6045–57.
86. Mochizuki, A., Yahara, K., Kobayashi, I. and Iwasa, Y. 2006, Genetic addiction: selfish gene's strategy for symbiosis in the genome, *Genetics*, **172**, 1309–23.
87. Fukuyo, M., Sasaki, A. and Kobayashi, I. 2012, Success of a suicidal defense strategy against infection in a structured habitat, *Sci. Rep*., **2**, 238.
88. Pleška, M., Qian, L., Okura, R., et al. 2016, Bacterial autoimmunity due to a restriction-modification system, *Curr. Biol*., **26**, 404–9.
89. Vasu, K., Nagamalleswari, E. and Nagaraja, V. 2012, Promiscuous restriction is a cellular defense strategy that confers fitness advantage to bacteria, *Proc. Natl Acad. Sci. U.S.A*., **109**, E1287–93.
90. Wei, H., Therrien, C., Blanchard, A., Guan, S. and Zhu, Z. 2008, The Fidelity Index provides a systematic quantitation of star activity of DNA restriction endonucleases, *Nucleic Acids Res*., **36**, e50.
91. Kamps-Hughes, N., Quimby, A., Zhu, Z. and Johnson, E.A. 2013, Massively parallel characterization of restriction endonucleases, *Nucleic Acids Res*., **41**, e119.
92. Bouma, J.E. and Lenski, R.E. 1988, Evolution of a bacteria/plasmid association, *Nature*, **335**, 351–2.
93. Oliveira, P.H., Touchon, M. and Rocha, E.P. 2014, The interplay of restriction-modification systems with mobile genetic elements and their prokaryotic hosts, *Nucleic Acids Res*., **42**, 10618–31.
94. Roberts, R.J., Vincze, T., Posfai, J. and Macelis, D. 2015, REBASE–a database for DNA restriction and modification: enzymes, genes and genomes, *Nucleic Acids Res*., **43**, D298–9.
95. Chinen, A., Naito, Y., Handa, N. and Kobayashi, I. 2000, Evolution of sequence recognition by restriction-modification enzymes: selective pressure for specificity decrease, *Mol. Biol. Evol*., **17**, 1610–9.
96. Ohno, S., Handa, N., Watanabe-Matsui, M., Takahashi, N. and Kobayashi, I. 2008, Maintenance forced by a restriction-modification system can be modulated by a region in its modification enzyme not essential for methyltransferase activity, *J. Bacteriol*., **190**, 2039–49.
97. Dewitt, S.K. and Adelberg, E.A. 1962, The occurrence of a genetic transposition in a strain of *Escherichia coli*, *Genetics*, **47**, 577–85.
98. Blattner, F.R., Plunkett, G., Bloch, C.A., et al. 1997, The complete genome sequence of *Escherichia coli* K-12, *Science*, **277**, 1453–62.
99. Fomenkov, A., Xiao, J.P., Dila, D., Raleigh, E. and Xu, S.Y. 1994, The 'endo-blue method' for direct cloning of restriction endonuclease genes in *E. coli*, *Nucleic Acids Res*., **22**, 2399–403.
100. McCool, J.D., Long, E., Petrosino, J.F., Sandler, H.A., Rosenberg, S.M. and Sandler, S.J. 2004, Measurement of SOS expression in individual *Escherichia coli* K-12 cells using fluorescence microscopy, *Mol. Microbiol*., **53**, 1343–57.
101. Stirling, C.J., Colloms, S.D., Collins, J.F., Szatmari, G. and Sherratt, D.J. 1989, xerB, an *Escherichia coli* gene required for plasmid ColE1 site-specific recombination, is identical to pepA, encoding aminopeptidase A, a protein with substantial similarity to bovine lens leucine aminopeptidase, *EMBO J*., **8**, 1623–7.
102. Flinn, H., Burke, M., Stirling, C.J. and Sherratt, D.J. 1989, Use of gene replacement to construct *Escherichia coli* strains carrying mutations in two genes required for stability of multicopy plasmids, *J. Bacteriol*., **171**, 2241–3.
103. Blakely, G., Colloms, S., May, G., Burke, M. and Sherratt, D. 1991, *Escherichia coli* XerC recombinase is required for chromosomal segregation at cell division, *New Biol*., **3**, 789–98.
104. Blakely, G., May, G., McCulloch, R., et al. 1993, Two related recombinases are required for site-specific recombination at dif and cer in *E. coli* K12, *Cell*, **75**, 351–61.