Evidence for an evolutionary antagonism between Mrr and Type III modification systems

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Received March 9, 2011; Revised March 23, 2011; Accepted March 24, 2011

ABSTRACT

The Mrr protein of Escherichia coli is a laterally acquired Type IV restriction endonuclease with specificity for methylated DNA. While Mrr nuclease activity can be elicited by high-pressure stress in E. coli MG1655, its (over)expression per se does not confer any obvious toxicity. In this study, however, we discovered that Mrr of E. coli MG1655 causes distinct genotoxicity when expressed in Salmonella typhimurium LT2. Genetic screening enabled us to contribute this toxicity entirely to the presence of the endogenous Type III restriction modification system (StyLTI) of S. typhimurium LT2. The StyLTI system consists of the Mod DNA methyltransferase and the Res restriction endonuclease, and we revealed that expression of the LT2 mod gene was sufficient to trigger Mrr activity in E. coli MG1655. Moreover, we could demonstrate that horizontal acquisition of the MG1655 mrr locus can drive the loss of endogenous Mod functionality present in S. typhimurium LT2 and E. coli ED1a, and observed a strong anti-correlation between close homologues of MG1655 mrr and LT2 mod in the genome database. This apparent evolutionary antagonism is further discussed in the light of a possible role for Mrr as defense mechanism against the establishment of epigenetic regulation by foreign DNA methyltransferases.

INTRODUCTION

In general, DNA restriction endonucleases (REases) team up with DNA methyltransferases (MTases) to form dedicated restriction modification (RM) systems, in which the MTase modifies DNA at specific sequences in order to protect it from cleavage by the cognate REase (1,2). Because of this interplay, bacterial RM systems behave as a primitive immune system in which the REase restricts the cellular entry of DNA lacking the proper methylation signature. As such, it can protect the host against deleterious phage infection or lateral acquisition of foreign DNA (3). However, as the genes encoding such RM systems are typically closely linked and often organized into an operon, they can also behave as selfish addiction modules that counteract their loss from the cell by post-segregational killing (4,5). In this phenomenon, siblings that lost the RM module will experience a decline in corresponding MTase activity, and eventually fail to protect all genomic target sites from cleavage by the residual cognate REase.

Depending on their operational characteristics, RM systems can be categorized into three main types (Type I–III) (6). As such, Type I systems are unique in requiring a dedicated specificity protein that directs activity of the corresponding MTase and REase to the target sequence, with cleavage itself occurring at variable distances from this site. In contrast, Type II MTase and REase pairs independently recognize the same target sequence and most often function as separate entities, with cleavage occurring at a defined position within or close to the recognition site.

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Finally, Type III systems display specificity for a short asymmetric recognition sequence, and cleave at a characteristic distance away from it. Importantly, binding to the target site is strictly mediated by the MTase, so that restriction is performed by a MTase–REase complex rather than by the REase alone. While all RM systems require AdoMet for donation of the methyl group, Type I and III systems additionally depend on ATP for their activity (6).

Interestingly, however, a small number of REases operate in the absence of a cognate MTase and have been grouped into a fourth category (Type IV). These REases invariably bear a distinct specificity for modified (e.g. methylated, hydroxymethylated or glucosyl-hydroxymethylated) DNA, although their recognition site is often poorly defined (6). Well-known examples of such solitary and modification-dependent REases in *Escherichia coli* are McrA, McrBC and Mrr, of which the corresponding genes are typically located on laterally acquired genetic elements such as e14 (*mcrA*) and the ‘Immigration Control Region’ (*mcrBC* and *mrr*) (7–9).

While McrA and McrBC are relatively well-characterized, and shown to be involved in restricting phage infection (10–14), the physiological role of Mrr in the cell has been less obvious so far. Recently, however, our interest in Mrr was raised as it appears to be a driving force of filamentation and cellular filamentation and prophase induction caused by sub-lethal high-pressure shock in *E. coli* K12 were the result of a RecBCD-dependent SOS response that was instigated by Mrr endonuclease activity (15–18). Unfortunately, it so far remains unclear how exactly Mrr becomes activated by a physical stress such as high pressure. Further study, however, revealed that not all Mrr homologues behave the same way, and the Mrr protein encoded by *Salmonella typhimurium* LT2, in fact, could not be activated by high-pressure shock (19).

The current study starts with the serendipitous observation that the Mrr protein of *E. coli* MG1655 causes distinct genotoxicity when expressed in *S. typhimurium* LT2, and eventually results in postulating an evolutionary antagonism between Mrr and Type III MTases.

**MATERIALS AND METHODS**

**Strains and construction of mutants**

*Salmonella typhimurium* LT2 (20, laboratory collection), *Escherichia coli* K12 MG1655 (21, laboratory collection) and *E. coli* ED1A (22, kindly provided by Eric Denamur, Institut Pasteur, Paris, France) were used as parental strains in this study.

An LT2 derivative cured of its four active prophages (i.e. strain MA8508 constructed by Lemire and Bossi, manuscript submitted for publication) was kindly provided by Lionello Bossi (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France), and is referred to as LT2Δsfi in this study. Strain LT2K2 is a derivative of LT2 and harbors a MudK (23) based translational lacZ fusion to the SOS-responsive *STM0912* gene, and its use as an SOS indicator strain has been validated previously (19). LT2 recA was constructed by cotransducing the *recA1* and *sfiA*-202::Tn10 alleles from TT521 (kindly provided by John Roth, University of California at Davis, USA) with P22 *HT105/1 int-201* (24). In strain LT2 ΔStyLTI, the StyLTI *mod res* operon was deleted based on the protocol of Datsenko and Wanner (25), and using an ampiclon prepared on pKD4 with the following primers: 5′-ATAAGAAATTTGATGGCTGCCACCTAAATTATCAACGGAGCGAAGATATCGTGTA-3′ and 5′-ATTITATATGGTCTATCCAAAAAGATACCCGTTAAGGTTCATTCAAAAAGATAACCGTTAAAGGTTCATTCAAAAAGATAACCGTTAAGGTTCATTCAAAAAGATAACCGTTAAGGTTCATTCAAAAAGATAACCGTTAAGGTTCATTCAAAAAGATAACCGTTAAGGTTCATTCAAAAAGATAACCGTTAAGGTTCATTCAAAAAGATAACCGTTAAGGTTCATTCAAAAAGATAACCGTTA-3′. This procedure replaced the *mod res* operon with an *ftr* flanked kanamycin resistance cassette, which could subsequently be excised by transiently equipping this strain with plasmid pCP20 expressing the Flp site-specific recombinase (26). Strain LT2K2 Δmod res was subsequently constructed by transducing *STM0912*:MudK to LT2 Δmod res using P22 *HT105/1 int-201*.

Strain MG1655 *mrr* harbors an *mrr*:Tn5 allele, which was obtained through an *in vitro* transposomics procedure using the EZ-Tn5 transposome kit (Epigentec, Landgraaf, The Netherlands) and an ampiclon of the MG1655 gene (*mrr*MG1655). The latter was obtained by PCR using the following primers: 5′-CGATTCTAGAGCTCGAAGGATGTACTATGACG-3′ and 5′-CGATAAGCTTGCGTGTGGCGGGTGTGGAGG-3′. The resulting *mrr*:Tn5 allele confers kanamycin resistance and was exchanged with the wild-type *mrr* allele according to the protocol of Datsenko and Wanner (25). Strain MG1655 λsfiA::lacZ was constructed by lysogenizing strain DJ624 (MG1655 ΔlacX74 mal::lacIq) (27) with λsfiA::lacZ, originating from CSH50 λsfiA::lacZ (28). Subsequently, Strain MG1655 λsfiA::lacZ *mrr* was constructed by transducing the *mrr*:Tn5 allele from MG1655 *mrr* into MG1655 λsfiA::lacZ with P1vir (29).

Strains were transformed with the necessary plasmids by electroporation, while curing of temperature-sensitive plasmids, such as pKD46 and pCP20, 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.

**Growth conditions**

Stationary phase cultures were obtained by growth in lysogeny broth (LB) (30) for 21 h at 37°C under well-aerated conditions. Late exponential phase cultures in turn were prepared by diluting stationary phase cultures 1/100 or 1/1000 in fresh pre-warmed LB, and allowing further incubation at 37°C until OD600 = 0.6 as described earlier (31). When appropriate, the following chemicals (Applichem, Darmstadt, Germany) were added to the growth medium at the indicated final concentrations: ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), glucose (0.2 or 0.02 %) and arabinose (0.2 or 0.02%).

Where indicated, quantitative measurement of growth was performed with either a Multiskan Ascent reader (Thermo, Aalst, Belgium) or a Bioscreen C Microbiology
Construction of plasmids

Plasmid pFPV25-P~BAD~mod^LT2 is derived from the pFPV25 backbone (32), confers ampicillin resistance and conditionally expresses Mod^LT2 in the presence of arabinose. For its construction, an amplicon of the mod^LT2 allele was first obtained by PCR on genomic DNA of LT2, using the following primers: 5'-AACGCTTAGATACTCATGTGG -3' and 5'- CGTAAAGCCTTA TCTCCCTCTTAAACG -3'. Subsequently, this amplicon was digested with XbaI and HindIII, and cloned in pFPV25-P~BAD~mod^ED1A. Plasmid pFPV25-P~BAD~mod^ED1A was constructed by preparing a PCR amplicon of the mod^ED1A allele from genomic DNA of ED1A, using the following primers: 5'- ACACCTTAGATACTCATGTGG TACGTGGA -3' and 5'- CGTAAAGCCTTA TCTCCCTCTTAAACG -3'. Subsequently, this amplicon was digested with XbaI and PstI, and cloned in pFPV25-P~BAD~mod^ED1A. Plasmid pFPV25-P~BAD~mod^ED1A was constructed similarly, but using the mrrMG1655 amplicon obtained as described above. Plasmid pFPV25-P~BAD~mod^LT2 was constructed earlier in a similar way (19). Where necessary, plasmid pFPV25-P~BAD~ served as a control, and was obtained by removing the gfp gene from pAA100 with XbaI and HindIII, and reclosing the resulting fragment by self-ligation after blunting.

Plasmid pACYC184-P~BAD~mrr^MG1655 is derived from the pACYC184 backbone (33), confers chloramphenicol resistance and conditionally expresses Mrr^MG1655 in the presence of arabinose. For its construction, a fragment encoding P~BAD~mrr^MG1655 was cut out from the pFPV25-P~BAD~mrr^MG1655 vector with EcoRI and HindIII, and subsequently blunt-ended. This fragment was then ligated into the pACYC184 vector, which was first opened with HindIII and BamHI and blunt-ended, to yield pACYC184-P~BAD~mrr^MG1655. Plasmid pAA810, which is also pACYC184 based, was constructed earlier (18) and harbors the mrr^MG1655 locus under the control of its own promoter.

Screening for suppression of Mrr^MG1655 lethality in LT2 recA

After we established that pACYC184-P~BAD~mrr^MG1655 conferred severe lethality in LT2 recA when plated on M9 minimal medium (30) containing 0.2 % arabinose, two different screens were initiated to find suppressors of this lethality. In a first screen, LT2 recA pACYC184-P~BAD~mrr^MG1655 was transformed with a library containing random fragments of MG1655 genomic DNA cloned in pBR322 (kindly obtained from Susan Gottesman, National Institutes of Health, Bethesda, USA). In a second screen, a random MudJ knock-out library was constructed in LT2 recA pACYC184-P~BAD~mrr^MG1655 using the MudJ transposon delivery system described by Hughes and Roth (23). Next, the corresponding plasmid and transposon libraries were plated on M9 medium complemented with 0.2% arabinose and appropriate antibiotics, and clones able to grow on such plates were isolated and purified. Subsequently, the corresponding pBR322-plasmids were transformed to a fresh LT2 recA strain, while the corresponding transposon insertions were transduced to a fresh LT2 pACYC184-P~BAD~mrr^MG1655 strain after which the recA allele was co-transduced into the resulting strain as described above. These resulting strains were examined to check whether the Mrr^MG1655 resistant phenotype could be confirmed.

One valid MudJ clone arose from this selection procedure, and its insertion site was determined by subcloning the chromosome of the corresponding mutant in pUC18 (34) and selecting for the kanamycin resistance marker encoded on the MudJ transposon.

Measuring of SOS induction and DNA damage

SOS induction was measured via standard Miller assays (35), using LT2K2 or MG1655 kshA::lacZ-based indicator strains. For the biochemical detection of DNA breaks and their distribution throughout a population, terminal deoxynucleotidyl transferase dUTP nick end labeling (i.e. TUNEL assay; in situ Cell Death Detection Kit, Fluorescein, Roche Mannheim, Germany) was used, based on the manufacturer’s instructions and the amendments published earlier (36,37). Briefly, 1 ml of a culture was washed two times with phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 9.6 mM Na2HPO4, pH 7.3), and fixed with ice-cold 4% paraformaldehyde for 30 min at room temperature. Cells were then pelleted and resuspended in ice-cold permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) and incubated for 2 min at 4°C. Again cells were washed one time with PBS and labeled using TUNEL reagents (Guava Easy Cyte Plus, Guava Technologies, Inc. Hayward, CA, USA). To generate a positive control, an aliquot of fixed and permeabilized control cells was first exposed to the blunt 4-cutter BsuRI for 1 h at 37°C, before being washed and labeled with TUNEL reagents.

Constructing the phylogenetic tree of E. coli and S. enterica strains

To construct the phylogenetic tree of E. coli and S. enterica strains, all full-length Escherichia and Salmonella 16 S rRNA gene sequences were downloaded from the Greengenes database core set (38) in the FASTA aligned format. Next, E. coli ED1A 16 S rRNA gene sequence was identified using NCBI nblast (with E. coli CFT073 16 S rRNA as a seed) and added manually to the dataset. Gene sequences that could not be successfully aligned originated from partially sequenced genomes or were duplicative, and were removed from the dataset. Next, the dataset was re-aligned using Greengenes (38), which aligns 16 S rRNA gene sequences to 7682 characters
full-length gene templates. All thresholds were kept at default values. Finally, based on the alignment, the phylogenetic tree was calculated using MEGA 4.0 (39) employing the Minimum Evolution method and assuming a Jukes–Cantor model of nucleotide substitution. Bootstrap values based on 1000 replications are listed as percentages at the branching points.

Calculating the anti-correlation of Mrr<sub>MG1655</sub> and Mod<sub>LTL2</sub> homologues

Sequences of <i>E. coli</i> Mrr (gi: 127320) and <i>S. typhimurium</i> Mod (gi: 300193) were used as queries for PSI-BLAST (40) searches (<i>E</i>-value ≤ 1e⁻³) against the nr database using default parameters and run until convergence. We retrieved 2001 Mrr and 6878 Mod sequences. After removing sequences from partially sequenced genomes and redundant sequences, we performed sequence clustering based on pair-wise BLAST similarity scores, using Cluster Analysis of Sequences (CLANS) (41). The clustering was completed at <i>P</i> = 0.012 for Mrr and <i>P</i> = 0.004 for Mod, respectively. The reported <i>P</i>-values give a well-resolved separation of multiple distinct clusters. The <i>P</i>-value was chosen empirically, given the <i>P</i>-value plot for each data set, which shows a histogram of the number of sequences for each <i>E</i>-value below a given value. For example, a cut-off of 0.004 will exclude connections worse than 0.004. Clusters are thought of as robust if small changes in cut-off values do not result in major changes in their content. Next, we extracted members of clusters containing the Mrr query sequence (gi: 127320) and the Mod query sequence (gi: 300193) for correlation analysis. At the end, we obtained 75 Mrr<sub>MG1655</sub> and 211 Mod<sub>LTL2</sub> homologues derived from 272 fully sequenced genomes and eliminated duplicate species to obtain 45 Mrr<sub>MG1655</sub> and 156 Mod<sub>LTL2</sub> homologues derived from 192 fully sequenced genomes.

The Pearson <i>r</i> correlation coefficient measures the degree to which values of two variables are linearly related to each other. It is defined as the covariance of two variables divided by the product of their standard deviations and was calculated for the Mrr<sub>MG1655</sub> and Mod<sub>LTL2</sub> families using STATISTICA 8 (StatSoft, Inc., Tulsa, OK, USA). We also determined the probability that the observed correlation is real and not a chance occurrence. The obtained correlation of −0.52 for 45 Mrr<sub>MG1655</sub> and 156 Mod<sub>LTL2</sub> homologues, derived from 192 fully sequenced genomes, is less than the critical value for <i>df</i> = 190, <i>α</i> = 0.05, two-tailed test with <i>P</i> < 0.0001.

Determination of the horizontal transfer index for homologs of mrr<sup>MG1655</sup> and mod<sup>LTL2</sup>

Nakamura et al. (42) have published an analysis of 116 completely sequenced propharytic genomes, in which they calculated an index of potential distant horizontal transfer for all genes, by comparing the frequency of ‘words’ of pentanucleotide length within each gene with the average word frequency of the entire genome. This index can be used to detect only recent transfers of genes from species with distinct gene sequence compositions, because genes transferred between similar genomes usually display similar sequence compositions and genes transferred a long time ago tend to accommodate their sequence composition to that of the new host. We examined the horizontal transfer index of genes encoding Mrr<sub>MG1655</sub> and Mod<sub>LTL2</sub> homologues in the updated set of 165 genomes (Yoji Nakamura and Takashi Gojobori, personal communication).

RESULTS

Expression of Mrr<sup>MG1655</sup> is harmless in <i>E. coli</i> MG1655 but genotoxic in <i>S. typhimurium</i> LT2

Previously, we have shown that, in contrast to MG1655, LT2 is unable to mount the SOS response after high-pressure shock (19). However, during our initial attempts to possibly restore this feature by complementing LT2 with a plasmid encoding the mrr<sup>MG1655</sup> gene under the control of its own promoter (i.e. pAA810), we obtained colonies with a sick translucent appearance. Moreover, when transformants were grown in liquid broth, lysis of the culture (Figure 1A) and concomitant accumulation of cellular debris could be clearly observed.

As the genome of LT2 contains four functional prophages (20), we postulated that cell lysis was caused by prophage activation due to Mrr<sup>MG1655</sup>-mediated DNA damage. Upon further investigation, an LT2 mutant deprived of its active prophages (LT2Δφ) indeed failed to show lysis (Figure 1B). Moreover, we could clearly detect SOS induction (Figure 1C) and even confirm physical DNA damage (Figure 1D) upon expression of Mrr<sup>MG1655</sup> in LT2, both revealing the severe genotoxicity of this protein in LT2.

Despite its constitutive toxicity in LT2, it is important to note that Mrr<sup>MG1655</sup> was harmless upon similar ectopic expression in its natural MG1655 host (data not shown), unless such strains were exposed to high-pressure shock (18). We, therefore, assumed this surprising dissimilarity to stem from differences in the physiology of both strains, resulting either in dedicated mechanisms of Mrr<sup>MG1655</sup>-restriction alleviation in MG1655 or dedicated mechanisms of Mrr<sup>MG1655</sup> activation in LT2.

The endogenous StyLTI Type III restriction modification system is responsible for Mrr<sup>MG1655</sup> toxicity in <i>S. typhimurium</i> LT2

In order to find out why Mrr<sup>MG1655</sup> behaves so differently in closely related species, a selection scheme was devised in which we tried to suppress the lethality of Mrr<sup>MG1655</sup> in LT2. First of all, delivery of Mrr<sup>MG1655</sup> was made dependent on the presence of arabinose (using pACYC184-P<sub>BAD</sub>-<sub>mrr<sup>MG1655</sup></sub>), in order to clearly define the onset of genotoxicity and avoid trivial suppressor mutations in the mrr<sup>MG1655</sup> locus. As such, conditional expression of Mrr<sup>MG1655</sup> reduced viability of LT2 wild-type with almost 99.99% (i.e. ca. 4-log reduction), although this lethality could be mitigated in the LT2Δφ strain (Figure 2). Subsequently, in order to avoid picking up suppressor mutants that would trivially be affected in prophage induction, we used an LT2 recA mutant as the
latter does not support prophage activation but proved to be similarly hypersensitive to MrrMG1655 because of its inability to repair DNA damage (Figure 2). Finally, suppression of MrrMG1655 toxicity in LT2 recA pACYC184–P_BAD–mrr MG1655 was attempted by introducing (i) random transposon knock-outs or (ii) a multi-copy plasmid library of the MG1655 genome. As such, we hoped to identify cellular functions that either trigger or mitigate MrrMG1655 activity in LT2, respectively.

Of both strategies, only the knock-out approach resulted in a bona fide MrrMG1655 resistant mutant, and subcloning enabled us to map its transposon insertion site to the LT2 res gene (Figure 2). This gene is located immediately downstream of the mod gene, and the mod res operon encodes the Type III StyLTI restriction–modification system of LT2. In order to unequivocally corroborate the interference of the StyLTI system with MrrMG1655, clean Δmod, Δres and Δmod Δres mutants were constructed de novo in wild-type LT2 and examined. Confirmingly, MrrMG1655 lethality (Figure 3A) and genotoxicity (Figure 3B) proved to be completely abolished in LT2 Δmod and Δmod Δres mutants, and to be greatly attenuated in an LT2 Δres mutant. These results clearly delineate the Type III StyLTI system as the sole feature responsible for constitutive MrrMG1655 toxicity in LT2.

**Functional incompatibility between ModLT2 and MrrMG1655**

Based on the above results, it was postulated that ModLT2 MTase activity would render the chromosome susceptible...
to degradation by Mrr<sup>MG1655</sup>, and the corresponding genes were conditionally co-expressed in MG1655 <i>mrr</i>. While expression of <i>mod</i><sup>LT2</sup> or <i>mrr</i><sup>MG1655</sup> alone was completely harmless in this background, their co-expression resulted in poor growth (data not shown) and massive SOS induction (Figure 4). These data confirm the apparent incompatibility between both proteins, and identified Mod<sup>LT2</sup> activity as instigator of Mrr<sup>MG1655</sup> restriction.

In order to examine whether this functional incompatibility could also extend into mutual exclusiveness between <i>mrr</i><sup>MG1655</sup> and <i>mod</i><sup>LT2</sup> loci, LT2 was equipped with pAA810 and 10 independent clones were followed for a number of generations. After only two passages, cultures grew without lysis (Figure 5A) or the accumulation of cellular debris, and gave rise to healthy colonies upon plating. Importantly, four out of these 10 independent suppressor mutants carried mutations in <i>mod</i><sup>LT2</sup> (Figure 5B), while others were affected in the <i>mrr</i><sup>MG1655</sup> locus encoded on pAA810. Interestingly, two of the compromised <i>mod</i><sup>LT2</sup> alleles suffered frame-shift mutations within short homopolymeric tracts present in the open reading frame (Figure 5B). These data indicate that Mrr<sup>MG1655</sup> and Mod<sup>LT2</sup> activities are mutually exclusive, and that acquisition of one could lead to functional inactivation of the other.
In the same context, it was noteworthy to find that close homologues of \textit{mrr} \textit{MG1655} and \textit{mod} \textit{LT2} often seem to co-exist within \textit{S. enterica} (Figure 6), which could imply the functional loss of one of these alleles. Indeed, most \textit{S. enterica} \textit{mrr} alleles very closely resemble that of strain LT2, and we were unable to observe any \textit{ModLT2}-dependent (or in fact any) genotoxicity upon deliberate over-expression of \textit{Mrr} \textit{LT2} in LT2 (data not shown), further supporting the hypothesis that the latter protein could have been degenerated as the result of functional counter-selection by an active \textit{Mod} in \textit{S. enterica}. Despite recent advancements in the structure–function relationship of \textit{Mrr} proteins (43), we were unable to pinpoint the exact reason for the inactivity of \textit{Mrr} \textit{LT2}.

Figure 6. Minimal evolution phylogenetic tree of 16S rRNA derived from fully sequenced \textit{Escherichia coli} and \textit{Salmonella enterica} genomes. The number at each node corresponds to the percent bootstrap value (1.000 bootstraps). Additionally, NCBI Taxonomy ID as well as \textit{Mrr} and \textit{Mod} protein content is indicated in the lineage of each strain. The branches are colored blue for genomes with \textit{Mrr} homologues, red with \textit{Mod} homologues, green with both \textit{Mrr}/\textit{Mod} homologues, and yellow with no \textit{Mrr}/\textit{Mod} homologues.

Figure 5. Spontaneous alleviation of \textit{Mrr} \textit{MG1655}-mediated toxicity. (A) Typical restoration of normal growth of LT2 pAA810 during consecutive passages (1/100 dilution at indicated time points) in LB medium. Representative data are shown. (B) Mutations identified in the \textit{modLT2} alleles of four independent LT2 pAA810 mutants in which \textit{Mrr} \textit{MG1655} toxicity was suppressed. Numbers indicate the position of the base at the beginning and end of the displayed wild-type (i.e. upper) sequence with respect to the start of the open reading frame. Asterisks highlight the actual deletions or substitution in each of the four alleles (i.e. lower sequence).
Functional incompatibility between ModED1A and MrrMG1655

When looking at the phylogenetic distribution in _E. coli_ (Figure 6), on the other hand, it seems that homologues of _mrrMG1655_ and _modLT2_ are frequently lost or acquired throughout evolution. Moreover, in agreement with their proposed incompatibility, there is a noticeable anti-correlation between both loci, and some _E. coli_ strains (e.g. ED1A), in fact, lack an _mrr_ allele while harboring a _mod_ allele instead. Importantly, when the _modED1A_ gene was cloned and co-expressed with _mrrMG1655_ in _MG1655_ _mrr_, we observed clear evidence for genotoxicity (Figure 7). This indicates that, despite bearing only 34% amino acid sequence identity with _ModLT2_, _ModED1A_ is also capable of rendering the chromosome susceptible for cleavage by MrrMG1655.

Furthermore, similar to LT2, ED1A also became sick upon acquisition of pAA810 (carrying the _mrrMG1655_ locus), and readily yielded suppressor mutants (data not shown). Interestingly, while half (i.e. five out of 10) of these mutants were affected in the _mrrMG1655_ locus on pAA810, the other half failed to yield an amplicon of the _modED1A_ gene by PCR. In this context, it is noteworthy that the _modED1A_ locus is located near a prophage integrase gene, perhaps indicating that it forms part of a mobile genetic element that can easily be excised from the chromosome.

Evolutionary antagonism between close homologues of MrrMG1655 and ModLT2

Spurred by the clear asymmetric phylogenetic distribution of _mrrMG1655_ and _modLT2_ homologs within _E. coli_ and _S. enterica_ (Figure 6), we subsequently examined their horizontal transfer index throughout 165 sequenced prokaryotic genomes according to the method of Nakamura _et al._ (42). Although this index only permits detection of recent gene transfers from species with distinct gene sequences, we nevertheless found that 11% (2/18) of MrrMG1655 homologues and 28% (9/32) ModLT2 sequence compositions, we nevertheless found that 11% (2/18) of MrrMG1655 homologues and 28% (9/32) ModLT2 homologues were most likely the subject of such recent horizontal transfer. Accordingly, we inferred that close homologues of _mrrMG1655_ and _modLT2_ could more generally act as mobile elements, and subsequently wondered whether the mutual exclusiveness of both genes would extend beyond strains of _E. coli_ and _S. enterica_. To approach this question, we searched 272 completely sequenced bacterial and archaeal genomes, and listed those in which MrrMG1655 and/or ModLT2 homologues were present (Figure 8). Using this list, the Pearson r correlation coefficient was calculated for 192 of these genomes (with one sampling per genome) and shown to be −0.52, which indicated a strong and statistically significant (_P_ < 0.0001) anti-correlation between both loci. As such, the presence of an Mrr homolog tends to be commonly paired with the absence of a Mod homolog in the same genome, and _vice versa_.

DISCUSSION

During our attempts to rescue the high-pressure induced SOS response in _S. typhimurium_ LT2 by introducing MrrMG1655 of _E. coli_ MG1655, we observed that the presence of this Type IV REase conferred constitutive (i.e. without the need for activation by high pressure) genotoxicity in LT2, while a similar toxicity was totally absent in MG1655. Since our knowledge about Mrr functionality is still limited and fragmentary, we decided to dissect the molecular basis of this apparent discrepancy between its impact on MG1655 and LT2. As such, we were able to show that the observed genotoxicity was elicited by the activity of a Type III MTase present in LT2 (i.e. ModLT2 of the StyLTI system), which most likely makes the chromosome susceptible to degradation by MrrMG1655. This feature, however, was not limited to ModLT2 as another Type III MTase encoded by _E. coli_ ED1A (i.e. ModED1A) could similarly elicit MrrMG1655 activity. Furthermore, because of this functional antagonism, loci encoding MrrMG1655 and ModLT2/ED1A were shown to readily counter-select each other. In the same context, we revealed a strong and more general anti-correlation between close homologues of _mrrMG1655_ and _modLT2_ in the genome database, suggesting that their incompatibility might well-extend beyond the genus of _Escherichia_ and _Salmonella_. As such, this study is the first to provide evidence for Type III MTases as natural triggers of Mrr activity.

The observed interference between MrrMG1655 and ModLT2/ED1A has a number of interesting repercussions. As such, Type III MTases only methylate one specific strand of its 5–6 bp asymmetric target sequence, indicating that MrrMG1655 is, in fact, able to restrict hemi-methylated DNA. Moreover, during the writing of this article, _in vitro_
evidence was published that revealed a similar activity for an Mrr-like REase (i.e. MspJ) from Mycobacterium sp JLS (44). In addition to hemi-methylation, all currently characterized Type III MTases seem to modify adenine (45), indicating that their activity remains undetected by other characterized Type IV REases (i.e. McrBC and McrA) which specifically target modified cytosines (46,47). Finally, our results also indicate that inactivation or deletion of the resLT2 gene greatly attenuates ModLT2-dependent MrrMG1655 toxicity in LT2. In turn, this might suggest that the expression and/or activity of ModLT2 could be affected by ResLT2, which challenges the current assumption that Type III MTases function independently of their corresponding REases (48).

Interestingly, their specificity for methylated or modified DNA has allowed the solitary Type IV REases to fulfill a number of different activities or roles in the cell. As such, it was demonstrated that McrA, McrBC and GmrSD are able to restrict invading phages that chemically disguise their chromosome with increasingly complex base modifications (8,49). Indeed, while McrA and McrBC are able to restrict DNA containing hydroxymethylcytosine (HMC), GmrSD has developed specificity for glucosylated HMC, and this differentiation is thought to reflect the evolutionary arms race between phage modification systems and the host REases. Most recently, however, Fukuda et al. (50) demonstrated that the deliberate introduction of Type II MTases sharing the RmC sequence specificity of McrBC could trigger cell death in E. coli by eliciting McrBC-dependent chromosome degradation, thereby counter-selecting the establishment or maintenance of the MTase. As a result, these authors postulated a novel function for Type IV REases in warding off epigenetic regulation by incoming MTases (50,51). The functional and evolutionary antagonism between Type IV Mrr and Type III Mod systems observed in this report seems to corroborate this hypothesis.

Moreover, aside from being subject to horizontal gene transfer, some Type III MTases have actually been identified as dedicated epigenetic regulators, and have recently been shown to direct the expression of a number of genes (including virulence genes) in species of Neisseria and Haemophilus (48,52,53). Interestingly, and fuelling their importance as epigenetic regulators, the coding sequence of many Type III MTases harbors homopolymeric tracts or tandem repeats, allowing frequent frame-shifts to occur and making their expression prone to phase variation at the translational level (48,52). In turn, this results in a phase variable ON/OFF switching of Mod-dependent regulons (i.e. phasevarions), which is believed to be a strategy that increases the adaptive potential of populations facing fluctuating environmental conditions. Although these phenomena have not been thoroughly investigated in...
S. enterica or E. coli, it should be noted that the corresponding genes contain a number of 6–7 bp homopolymeric tracts. Moreover, we have shown such tracts to support phase variation in the mod^{L2} gene upon functional counter-selection of its gene product by Mrr^{MG1655}. Furthermore, it is clear from the genome database that a number of S. enterica strains naturally contain out-of-frame mod alleles. The spoilage pathogen S. Gallinarum str. 287/91, for example, harbors an inactive mod allele because of a +1 frame shift in a poly-G tract. Whether Mod also contributes to epigenetic regulation in strains of E. coli and S. enterica still remains to be established.

Aside from the control of phasevarions, however, frequent phase variation of Mod activity would have a number of interesting consequences with respect to its antagonism with Mrr. As such, lateral acquisition of Mrr could readily select for a population phenotypically remaining in the OFF state by counteracting those cells in which the mod allele switches ON. On the other hand, transient OFF states might actually have evolved in order to allow safe horizontal passage or dissemination of mod alleles through strains exhibiting Mrr activity, thus mitigating restriction or cell death. Finally, one could even imagine that switching Mod activity in the presence of a cognate and functional Mrr would temporarily allow a small subpopulation to experience bursts of DNA damage and increased mutation rates, and to behave as transient mutators that can generate genotypic variability. The transient nature of increased mutation rates is essential, as constitutive mutators eventually succumb to the accumulation of deleterious random mutations (54). Obviously, the validity and relevance of the possible phenomena stated above require further investigation.

In summary, we present data supporting the functional interference and corresponding genomic incompatibility between Type IV Mrr and Type III Mod activity within E. coli and S. enterica, and extrapolate that these observations might extend to other species. Furthermore, this apparent antagonism fits within the recently emerging view of Type III MTases as phase variable epigenetic regulators and the hypothesis that Type IV REases might have evolved to counteract the establishment of specific genome methylation systems.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Régis Hallez for helpful discussions, and Kelly Hughes, Lionello Bossi, John Roth and Eric Denamur for sharing strains.

FUNDING

The Polish Ministry of Science and Higher Education (Grant POIG.02.03.00-00-003/09 to E.W.); The EU 7FP (Grant number 229676 to J.M.B.); Erasmus Mundus External Cooperation Window (EMECW13-Eurindia to A.G.); The Research Foundation - Flanders (FWO-Vlaanderen, 1.5.258.08N to A.A.); The K. U. Leuven Research Fund (CREA/09/017 to A.A.). Funding for open access charge: Partially waived by Oxford University Press.

Conflict of interest statement. None declared.

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