Mrr instigates the SOS response after high pressure stress in *Escherichia coli*

Abram Aertsen* and Chris W. Michiels

Laboratory of Food Microbiology, Katholieke Universiteit Leuven, Kasteelpark Arenberg 22, B-3001 Heverlee, Leuven, Belgium.

Summary

The bacterial SOS response is not only a vital reply to DNA damage but also constitutes an essential mechanism for the generation of genetic variability that in turn fuels adaptation and resistance development in bacterial populations. Despite the extensive depiction of the SOS regulon itself, its activation by stresses different from typical DNA damaging treatments remains poorly characterized. Recently, we reported the RecA- and LexA-dependent induction of the SOS response in *Escherichia coli* MG1655 after exposure to high hydrostatic pressure (HP, ∼100 MPa), a physical stress of which the cellular effects are not well known. We now found this HP mediated SOS response to depend on RecB and not on RecF, which is a strong indication for the involvement of double strand breaks. As the pressures used in this work are thermodynamically unable to break covalent bonds in DNA, we hypothesized the involvement of a cellular function or pathway in the formation of this lesion. A specialized screening allowed us to identify the cryptic type IV restriction endonuclease Mrr as the final effector of this pathway. The HP SOS response and its corresponding phenotypes could be entirely attributed to the HP triggered activation of Mrr restriction activity. Several spontaneously occurring alleles of *mrr*, incapable of triggering the HP-induced SOS response, were isolated and characterized. These results provide evidence for a specific pathway that transmits the perception of HP stress to induction of the SOS response and support a role for Mrr in bacterial stress physiology.

Introduction

The bacterial SOS response comprises an elaborate genetic stress regulon that is typically induced when cells suffer DNA damage (Friedberg *et al.*, 1995). Due to stalling and dissociation of the replication fork, DNA lesions will often lead to exposure of single-stranded DNA (ssDNA), which in turn is rapidly sensed and bound by RecA. The resulting nucleoprotein complex will activate RecA and lend it the ability to stimulate the autoproteolytic activity of LexA. As intact LexA binds to the promoter region of SOS genes and acts as a global repressor of the SOS regulon, its RecA-stimulated autocleavage will titrate the repressor away from its DNA binding site and allow transcription of the SOS regulon. The SOS regulon encodes proteins involved in nucleotide excision repair, recombinational repair or error prone DNA synthesis, which are necessary to repair the DNA damage or to facilitate DNA replication across the lesion (Friedberg *et al.*, 1995; Kuzminov, 1999). In addition, the SOS inducible SulA protein directly interacts with FtsZ to inhibit cell division in the presence of DNA damage (Huisman and D’Ari, 1981; Cordell *et al.*, 2003). Similar to the LexA repressor, the CI repressor protein encoded by lambdoid prophages, which maintains a lysogenic state, will cleave itself in the presence of activated RecA, leading to initiation of the lytic cycle and the production and release of phage particles (Roberts and Devoret, 1983).

It is well established that induction of the SOS response accounts for interesting microbial behaviour ranging from cell filamentation (Huisman and D’Ari, 1981) and the release of phage particles (Roberts and Devoret, 1983) to increased levels of mutagenesis (Caillet-Fauquet and Maenhaut-Michel, 1988; Walker, 1995; Radman, 1999), DNA rearrangements (Morel *et al.*, 1998), transposon excision and transposition (Kuan *et al.*, 1991; Eichenbaum and Livneh, 1998), and intra- and interspecies recombination (Matic *et al.*, 1995). This genetic impact is considered of major importance because it can be seen as a stress-induced strategy to generate genetic variability and as such increase the existing genetic repertoire of bacterial populations, enabling them to more quickly and adequately accommodate to their environment (Taddei *et al.*, 1997; Radman *et al.*, 2000; Yeiser *et al.*, 2002; Cirz *et al.*, 2005). In this context, it is of great interest to define conditions that lead to SOS induction and, in particular, to identify the exact cellular factors capable of eliciting this response. Indeed, opposed to the direct DNA lesions incurred by UV irradiation or exposure to genotoxins such as mitomycin C (MC), recent evidence suggests that several indigenous factors are also capable of mediating acti-
vation of the SOS response in the cell (reviewed by Aertsen and Michiels, 2005a). As such it was already shown that the SOS response is triggered in ageing colonies of E. coli, by a mechanism depending heavily on intracellular cAMP levels in addition to RecA and LexA (Taddei et al., 1995). More recently even specific gene functions were found to trigger the SOS response in E. coli cells experiencing defective cell wall synthesis instead of DNA damage (Miller et al., 2004). When exposed to β-lactam antibiotics, more specifically those acting on PBP3 (FtsI), compromised cell wall synthesis leads to the activation of the DpiBA two component signal transduction system, while the subsequent interaction of DpiA with the A+T-rich sequence of the chromosomal replication origin (oriC) affects chromosome replication and in turn activates the SOS response (Miller et al., 2003).

In the same vein we have recently observed a bona fide induction of the SOS response in E. coli MG1655 exposed to high pressure (HP; ∼100 MPa) (Aertsen et al., 2004a). HP represents an important environmental physical parameter in the biosphere, being even strictly required for the growth and survival of specific piezophilic bacteria in microbial deep-sea niches and in the deep subsurface (Yayanos, 1995; Bartlett, 2002). Although the importance of HP in the evolution of non-extremophiles such as E. coli may be considered questionable, exposure to HP nevertheless evokes interesting microbial behaviour and disruption of the HP-induced response may shed new light on the stress physiology of such organisms. Indeed, recent studies indicate that in E. coli MG1655 the genetic response towards HP stress not only combines features of both the heat and cold shock response (Welch et al., 1993; Aertsen et al., 2004b; Ishii et al., 2005), but also leads to a potent RecA- and LexA-dependent induction of the SOS response (Aertsen et al., 2004a). Comparable with classic UV irradiation, HP stress causes hyperfiliation in Lon deficient cells (Aertsen and Michiels, 2005b) and is capable of triggering induction of lambdoid prophages in lysogens of E. coli MG1655 (Aertsen et al., 2004a; 2005a).

As previously HP has never been associated with any form of DNA damage, we were interested in the actual molecular mechanism behind its activation of the SOS response. In this study we report evidence of a novel stress-induced pathway that links the physical perception of HP to the induction of a bona fide SOS response through activation of the Mrr restriction endonuclease.

Results

High pressure causes double strand breaks in E. coli MG1655

We have previously observed that the SOS induction by HP is RecA-dependent, implicating ssDNA mediated activation of the RecA protein (Aertsen et al., 2004a). In the cell ssDNA is rapidly covered by the single strand binding protein and, depending on the type of lesion, involves the intervention of either the RecFOR or RecBCD pathway to make it accessible to RecA (Kuzminov, 1999). In a first step towards elucidating the molecular mechanism behind HP SOS induction, we investigated whether the HP-mediated DNA lesion could be sensed by the RecFOR or the RecBCD complex, thus discriminating between a daughter strand gap (DSG) or a double strand break (DSB) respectively. MG1655 wild-type, ΔrecA, recF::Kn and recB::Kn were transformed with a transcriptional fusion to the recA promoter and subjected to HP, UV or MC treatment. Subsequently, recA promoter activity was followed by measuring green fluorescent protein (GFP) fluorescence in time (Fig. 1A). In RecF deficient strains, DSGs typically cause a delay in SOS induction that roughly corresponds with the time necessary for the DNA replication machinery to carry out a next round of replication and turning DSGs into DSBs, and as such into a recognizable substrate for the RecBCD system (Kuzminov, 1999). While the recF strain clearly illustrated such a delay (c. 30 min) for UV and MC treatment, no delay or attenuation was observed for the HP-induced SOS response when compared with the parent strain. On the other hand, while SOS induction in the recB strain was unaffected for UV and only partially attenuated for MC

![Fig. 1. A. Induction of a recA-gfp transcriptional promoter fusion by HP (● 100 MPa, 20°C, 15 min), UV (▲, 0.1 kJ m⁻²) or MC (▲, 2 µg ml⁻¹) in MG1655 wild-type, recA, recF and recB backgrounds compared with untreated control cells (□). B. Inactivation of late exponential phase cells of MG1655 wild-type, recA, recF and recB after HP (100 MPa, 20°C, 15 min) treatment. Inactivation is expressed as log₉ of the RF. For all strains the initial cell concentration was c. 5 x 10⁸ cfu ml⁻¹. Results are means ± standard deviations from three independent experiments.](image-url)
treatment, the absence of RecB completely abolished HP SOS induction. This indicates that, while UV irradiation predominantly gives rise to DSGs and MC causes lesions of both kinds (Courcelle et al., 1999; Keller et al., 2001), HP treatment seems to exclusively create DSBs. The latter observation is corroborated by inactivation experiments, showing a comparable increase in HP sensitivity for recA and recB strains (Fig. 1B), supporting the successive involvement of RecB and RecA in DNA repair and propagation of the HP SOS response.

Most importantly, the indication of DSBs as the sole HP-induced DNA lesion virtually eliminates the possibility of a direct pressure effect on DNA, because thermodynamically pressure is unable to break covalent bonds (Heremans, 1995; Balny et al., 2002), and points to the involvement of a specific cellular pathway that elicits SOS induction upon perception of HP stress.

**Induction of the HP SOS response proceeds through an exclusive genetic pathway**

To find evidence for such an HP-induced SOS pathway, we attempted to isolate spontaneous mutants of MG1655 that are specifically compromised in their ability to mount an SOS response to HP, but not to UV or MC. To achieve this, we tried to isolate spontaneous compensatory mutations in a MG1655 $\text{lon}::\text{Kn}$ background, which could rescue resistance of this strain to either HP or UV. As Lon deficient E. coli strains are compromised in their ability to degrade the SOS inducible cell division inhibitor SulA, the concomitant severe filamentation makes them hypersensitive towards SOS inducing treatments. The following growth based enrichment strategy was applied: a stationary phase culture of MG1655 $\text{lon}::\text{Kn}$ was subcultured and grown to late exponential phase, subsequently the cells were pressurized (100 MPa, 15 min, 20°C), directly diluted 100-fold in fresh Luria–Bertani broth (LB) and grown to stationary phase again (defined as one cycle), after which the procedure was repeated. A similar set-up was carried out for UV treatment (0.1 kJ m$^{-2}$). Typically four subsequent treatment cycles were necessary to obtain a culture in which spontaneous rescued mutants of MG1655 $\text{lon}::\text{Kn}$ took over the population, and both for HP and UV at least 10 of such enrichment experiments were independently carried out, leading to the isolation of a number of independently rescued $\text{lon}$ mutants with a history of exposure to either HP or UV (Fig. 2).

Subsequently the growth (OD$_{600}$) of individual rescued mutants after UV or HP shock was compared with each other and with the parental MG1655 $\text{lon}::\text{Kn}$. From these comparisons, it appeared that the rescued $\text{lon}$ mutants independently originating from the UV-based selection all had the same growth delay after UV (0.1 kJ m$^{-2}$) or HP (100 MPa, 15 min, 20°C) treatment (Fig. 2B), and were most probably rescued by a mutation directly compromising SulA function. Although the latter assumption was not further confirmed genetically, sulA mutants represent the predominant suppressors of $\text{lon}$ sensitivity towards typical SOS inducing treatment (George et al., 1975; Gayda et al., 1976). As this is a downstream adaptation (i.e. at the end of the cascade that makes $\text{lon}$ cells SOS-sensitive) that directly counteracts deleterious SulA accumulation, it also provides cross-resistance towards HP treatment. Most interestingly, however, the rescued $\text{lon}$ mutants originating from the HP-based selection could be sorted in two categories, occurring with comparable frequencies: (i) one category that besides HP resistance also displays cross-resistance towards UV treatment (Fig. 2C), and which presumably is similarly affected in SulA function as the UV selected mutants; and (ii) one category that was not only more, but also exclusively resistant to HP treatment but proved as sensitive to UV treatment as the parental $\text{lon}$ strain (Fig. 2D).

The latter category is of particular interest, as it provides evidence for a HP-induced SOS pathway that is fundamentally different from the UV-induced pathway.
However, the above observations stemmed from a growth enrichment selection process, and were therefore illustrated with OD$_{600}$ measurements. In order to corroborate this finding, it was necessary to examine whether in this latter category the SOS response was also exclusively blocked for HP treatment and not for other SOS inducers such as UV or MC. Therefore, isolated representative clones of each category were transformed with the recA-gfp reporter fusion and exposed to HP, UV and MC treatment. Compared with MG1655 lorn::Kn (Fig. 2A) the isolated rescued clones originating from UV selection (Fig. 2B) and their counterparts of category (i) originating from HP selection (Fig. 2C) are unaffected in SOS induction by HP, UV or MC, further confirming an unspecific and downstream adaptation, such as impairment of SulA activity. In contrast, the clones isolated from category (ii), originating from HP selection, still exhibited the SOS response triggered by UV or MC, but were unable to mount an SOS response towards HP (Fig. 2D).

The fact that these latter mutants could be isolated provides convincing evidence that the actual and primary trigger leading to the development of the SOS response by HP is fundamentally different from the DNA damage directly caused by UV or MC itself, and might instead be mediated by a cellular pathway, upstream of RecA activation, that links perception of HP stress to the generation of DSBs and subsequently to SOS induction.

**Mrr is the ultimate elictor of the HP SOS response**

Assuming such a specific cellular pathway at the basis of the HP-induced SOS response, we constructed a random transposon insertion library in MG1655 lorn::Kn to test whether some loss-of-function mutation could abolish this putative pathway and concomitantly the HP mediated SOS response, regardless of its induction by classic DNA damagers. Sixty-three pools, each containing 96 random insertion mutants in MG1655 lorn::Kn, were individually subjected to only two HP cycles, because it was important to avoid the selection of spontaneously occurring HP resistant mutants. Afterwards the pools were plated and survival was compared with the parental lorn strain after two HP cycles.

One of the pools showed increased resistance towards HP treatment after two HP cycles, indicating the presence of an insertion mutant capable of taking over the population. After purification of some of these survivors, their transposon insertion was P1 transduced to MG1655 lorn::Kn and subsequent experiments illustrated that the resulting mutants indeed displayed HP resistance while being equally UV-sensitive as MG1655 lorn::Kn. The transposon insertion site was mapped to the mrr gene, coding for a cryptic type IV restriction endonuclease that has been described to specifically act on methylated DNA (Heitman and Model, 1987), and the resulting allele is referred to as mrr::Cm (Fig. 5).

To independently validate whether mrr is indeed at the basis of the HP-induced SOS response, the following experiments were carried out. The mrr::Cm allele was P1 transduced to MG1655 wild-type and the resulting strain was transformed with the transcriptional fusion of the recA promoter to gfp. This strain was treated with HP (100 MPa, 15°C, UV (0.1 kJ m$^{-2}$) and MC (2 µg ml$^{-1}$), and 3 h following subsequent incubation at 37°C the induction of the SOS response was determined at the population level using FACS analysis (Fig. 3A). Corresponding to the induction profile shown in Fig. 3A, it is clear that the HP-induced SOS response was specifically abolished, while the responses induced by UV or MC remained unaffected. It should be noted that the specific absence of the HP SOS response was not trivially due to cell death, because HP treatment did not affect viability of MG1655 mrr::Cm (see below). Similarly, when mrr::Cm was transduced to MG1655 λ, it specifically abolished the HP induction of λ prophage, leaving its UV induction unaffected when compared with wild-type MG1655 λ (Fig. 3B).

In a subsequent experiment, the effect of Mrr deficiency on transient filamentation of wild-type cells, and on hyper-
filamentation of lon cells was compared after UV and HP treatment. When MG1655 wild-type is exposed to UV (0.1 kJ m$^{-2}$) or HP (100 MPa, 15 min, 20°C) and further incubated in rich medium, a typical cell elongation phenotype is observed, referred to as transient filamentation. Apparently, in both cases this type of filamentation is SulA independent (Gottesman et al., 1981; Aertsen and Michiels, 2005b). Interestingly, mrr inactivation specifically abolished this phenotype for HP treatment, leaving the transient filamentation following UV treatment unaffected (Fig. 4A). Similarly, the hyperfilamentation of HP treated lon mutants was completely absent when Mrr function was compromised (Fig. 4A). Viability determination of late exponential cultures treated with HP are in agreement with these microscopic observations, and the survival of lon mrr double mutants was c. 50-fold improved compared to lon mutants (Fig. 4B). Inactivation of mrr even slightly improved survival of wild-type cells (Fig. 4B) and abolished the lag phase specifically after HP treatment (data not shown, but similar to Fig. 2D). It should be noted that mrr inactivation had no effect on the typical filamentous growth at permissive pressures (<50 MPa), nor could it increase the growth permissive pressure in MG1655 (data not shown).

The discovery of an endonuclease, Mrr, as part of the pathway that connects HP stress to SOS induction is in perfect agreement with the HP-induced DSBs and points out Mrr as the essential effector of the HP-induced SOS pathway. Interestingly, a transcriptional promoter fusion of the mrr promoter to gfp (pAA820) did not display increased transcription in response to HP (100 MPa, 15 min, 20°C) (data not shown), suggesting that while Mrr is constitutively present in the cell it only acts as a nuclease after the perception of HP stress.

Characterization of spontaneous mrr alleles that rescued HP resistance in lon cells

To investigate whether other mutations besides those that abolish Mrr function could specifically rescue HP resistance in lon cells without affecting UV sensitivity, the mrr alleles of 10 independently isolated category (ii) mutants were amplified by proofreading polymerase chain reaction (PCR) and, in case an mrr amplicon was present, sequenced. Interestingly, we found that all 10 independent mutants were somehow affected in their mrr gene. Some spontaneous mrr alleles were even isolated more than once and seven distinct mutant types were identified, ranging from point mutants and small in frame deletions to gene disruptions and loss of the entire mrr gene (Fig. 5). When we transformed the mutants with pJH40 (kindly provided by Joseph Heitman, Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, USA), expressing the HhaII methylase gene that was shown to make the host DNA prone to Mrr cleavage thus reducing transformation efficiency (Heitman and Model, 1987), all mutants exhibited a transformation efficiency that was c. 50-fold increased when compared with MG1655 lon::Kn cells carrying the wild-type mrr gene (data not shown). This increased efficiency was identical to MG1655 lon::Kn mrr::Cm cells, indicating that all isolated spontaneous mrr alleles were non-functional or severely attenuated in Mrr activity.

The isolated point mutations were in amino acids 81 (mrrII), 82 (mrrIII and III) and 92 (mrrIV), all causing only a single amino acid change in a region of the Mrr protein that is different from the enzyme active site but that seems nevertheless to be essential for Mrr functionality. By contrast, the mrrVII allele encodes an Mrr protein from which the active site is almost surgically removed by an in-frame deletion, probably as the result of a recombination event at an 8 bp direct repeat (TCAGGCGC; Fig. 5) spanning the structural core of the catalytic domain as defined by Bujnicki and Rychlewski (2001). The mrrVII allele suffered an internal out-of-frame deletion resulting in a truncated protein. Finally, two mrr alleles (mrrV and VI) are

![Fig. 4. A. Photomicrographs of control, UV (0.1 kJ m$^{-2}$) and HP (100 MPa, 15 min, 20°C) treated cells of late exponential phase cultures of MG1655 wild-type, mrr::Cm, lon::Kn, and lon::Kn mrr::Cm incubated for 3 h at 37°C after treatment. B. Corresponding inactivation when cultures were plated after treatment. Inactivation is expressed as log$_{10}$ of the RF. For all strains the initial cell concentration was c. 5 × 10$^{8}$ cfu ml$^{-1}$. Results are means ± standard deviations from three independent experiments.](image-url)
As Mrr acts on methylated DNA (Heitman and Model, 1987; Waite-Rees et al., 1991), we wanted to examine whether deficiency in the three currently characterized methylases of *E. coli* (*dam*, *dcm* and *hsdRMS*) could perhaps abolish Mrr toxicity after HP treatment. However, when *E. coli* GM272 (*dam-3 dcm-6 hsdS21*) was transformed with pAA810 and pACYC184, and subjected to HP (100 MPa, 15 min, 20°C), GM272 (pAA810) proved equally hypersensitive towards HP treatment as the methylase proficient MG1655 (pAA810), and also displayed a c. 500-fold increased inactivation when compared with GM272 (pACYC184) (data not shown). This indicates the lack of involvement of at least these key methylases.

It should also be noted that in our attempts to select and isolate HP-resistant revertants of the hypersensitive MG1655 (pAA810) by several consecutive HP cycles, again only mutants were obtained with an inactivated *mrr* gene on the plasmid (data not shown).

**Discussion**

Besides being indispensable for the repair of DNA damage, the bacterial SOS response also provides a central motor for the generation of microbial diversity (Radman et al., 2000). By triggering mutagenesis, recombination and the lateral spread of genes, the SOS response allows for the inducible generation of genetic diversity that becomes available for selection in a given environment, eventually resulting in the rapid development of adaptation and resistance in a population (Taddei et al., 1997; Radman et al., 2000; Yeiser et al., 2002; Cirz et al., 2005). Although the SOS response is typically induced by physical (e.g. UV) or chemical (e.g. MC) stresses that directly affect DNA integrity, it was more recently demonstrated that exposure to β-lactam antibiotics, affecting cell wall synthesis instead of DNA integrity, enabled SOS induction. Importantly, and in line with the absence of direct

![Fig. 5. Schematic representation of wild-type *mrr* allele (light arrows) compared with *mrr*:Cm and 10 independently isolated spontaneous *mrr* mutant alleles (dark arrows) with indication of specific defects. Please note that *mrrX* and *mrrX* did not yield an *mrr* amplicon with PCR.](image)

**Mrr overexpression leads to extreme HP sensitivity**

To test whether an increase in the cellular Mrr levels in MG1655 would increase HP sensitivity, the wild-type *mrr* allele was cloned in pACYC184 (pAA810) and transformed to MG1655 wild-type. For comparison, *mrrI* (point mutant) and *mrrVII* (in-frame deletion mutant) were cloned the same way in pACYC184 (pAA811 and pAA812 respectively) and also transformed to MG1655 wild-type. While none of the *mrr* alleles affected UV sensitivity, only the wild-type *mrr* allele drastically increased HP sensitivity (c. 500-fold) when provided in multiple copies (Fig. 6).
DNA damage, an intricate genetic pathway, based on the two component signal transduction system DpaBA, was found to connect compromised cell wall synthesis to induction of the SOS response (Miller et al., 2004). In this study, we report evidence of another stress-induced pathway linking the physical perception of HP to the induction of a bona fide SOS response.

The RecA dependence of the HP-induced SOS response, which we demonstrated earlier clearly indicated involvement of ssDNA and an activated RecA nucleoprotein filament, but it was not clear how HP treatment would result in the formation of ssDNA (Aertsen et al., 2004a). As DNA duplexes are very pressure stable (Heremans, 1995; Balny et al., 2002), a direct dissociation of both DNA strands could be excluded at the pressure levels used in this work. DNA replication and transcription on the other hand, being dependent on multi-subunit protein complexes, are very pressure-sensitive (Erijman and Clegg, 1998; Bartlett, 2002) and made us consider the possibility that ssDNA could be formed as a result of stalling of the replication or transcription complexes (Aertsen et al., 2004a). However, this hypothesis proved unlikely as it recently became clear that some natural occurring E. coli isolates, such as the strains EDL933 (O157:H7) and H19 (O26:H11), did not exhibit HP SOS induction while displaying no defects in the SOS cascade triggered by direct DNA damagers such as UV or MC (Aertsen et al., 2005a). Therefore, in the current paper, we considered the possibility of DNA damage as a result of HP treatment.

We first investigated whether activation of RecA would occur through processing of a putative DNA lesion by the RecFOR or RecBCD complex, discriminating between a DSG and DSB respectively (Kuzminov, 1999). Interestingly, HP activation of the SOS response depended entirely on RecB (Fig. 1), indicating DSBs as trigger for the SOS response. Because of thermodynamic constraints, HP at the levels used in this work is incapable of breaking covalent bonds (Heremans, 1995; Balny et al., 2002), precluding the direct generation of DSBs by HP. This consideration, together with the fact that HP activation of the SOS response is not a common trait shared by all E. coli strains, lead us to speculate on the presence of an HP-activated cellular pathway still upstream of the RecB-dependent activation of RecA. In a subsequent step we were able to isolate spontaneous mutants in a MG1655 lon background exhibiting the same compromised HP SOS induction phenotype as was observed for EDL933 and H19 (i.e. deficient only in the SOS response triggered by HP, but not by UV or MC), suggesting that the ability to induce the HP SOS response indeed involves a genetic trait that can be selected against. A subsequent loss of function screen by random transposon mutagenesis resulted in the identification of the Mrr protein, an endogenous restriction endonuclease, as the likely final effector in translating the perception of HP into DSBs and concomitant RecB-dependent SOS induction. In agreement with our findings, mrr is naturally absent in E. coli EDL933 (Sibley and Raleigh, 2004) and, as preliminary PCR points out, seems also absent in E. coli H19 (data not shown), explaining the earlier reported lack of HP-induced SOS response in these strains (Aertsen et al., 2005a).

Due to the central role of Mrr in triggering the HP-induced SOS response, it is clear that, especially in Lon deficient backgrounds in which the SOS response is detrimental, HP treatments provide a strong selection for loss of Mrr function. Interestingly, this HP selection resulted exclusively in mutants directly affected in the mrr gene, i.e. no mutants were found with an intact mrr gene. The abundance and diversity of compromised mrr alleles that have spontaneously appeared during HP cycling is noteworthy in that it reflects the natural and overall competence and creativity of bacteria to disrupt the function of deleterious genes. Moreover, mrr alleles isolated by the HP selection strategy developed in this work will contribute to the dissection of the structure–function relation of the Mrr protein.

Interestingly, Mrr was originally discovered by Heitman and Model (1987) through its ability to restrict DNA with a foreign, but still cryptic, methylation pattern. As a result, Mrr was shown to induce the SOS response after the introduction and expression of certain foreign methylases such as HhaII and PstI in the cell. This specificity for methylated DNA is in contrast with the vast majority of restriction endonucleases that typically target unmodified DNA, and marks Mrr as a type IV restriction endonuclease together with McrA and McrBC (Roberts et al., 2003). In addition, together with hsdRMS (the type I EcoKI restriction modification system) and mcrrBC, mrr is located in the highly variable Immigration Control Region (ICR), a cassette-like and foreign genomic region characterized by a high density of encoded restriction systems (Sibley and Raleigh, 2004). Straightforwardly, a set of such diverse restriction systems might serve (i) to adequately attack incoming phage from different origins and bearing different methylation patterns, and (ii) to regulate lateral gene flow by cutting incoming DNA into smaller pieces while simultaneously inducing SOS-dependent recombination functions (Raleigh et al., 1989). However, this report is the first to demonstrate that, at least in the case of Mrr, restriction activity can be stress-induced even in the absence of foreign methylases or DNA transfer.

Normally, endogenous restriction activity is carefully diverted from the chromosome by protecting specific target sites with a methylation pattern and, under stress conditions where this self-protection might be transiently compromised as in the case of hemimethylated duplexes.
during DNA repair, even by elegant restriction alleviation mechanisms. Indeed, for the EcoKI system it was shown that the ClpXP protease will specifically cleave HsdR when the latter is associated with a transiently unmodified chromosomal site, thus preventing chromosome breakage (Makovets et al., 1999). It is therefore noteworthy that a specific stress somehow seems to allow chromosomal restriction by Mrr to occur. This may indicate that some *E. coli* strains have accommodated this foreign and potentially harmful restriction endonuclease as a stress response trigger. The mechanism, however, remains unclear. An increase in Mrr protein levels due to pressure treatment seems unlikely because a *mrr-gfp* transcriptional fusion did not respond to HP, nor did increasing *mrr* copy number by introduction of pAA810 have any obvious toxic effect on growth of MG1655 in the absence of HP treatment. A previous study by Waite-Rees et al. (1991), making use of specific antibodies against Mrr, also revealed a steady basal expression of Mrr, irrespective of the presence of several foreign methylases known to confer an Mrr-sensitive phenotype.

The apparent central role of Mrr in the HP-induced SOS response could still involve two scenarios. One possibility is that Mrr is the sole gene product linking HP perception to SOS induction. This would imply either a direct activation of Mrr by HP or a structural change in the genomic DNA that makes it susceptible to restriction. Alternatively, a putative Mrr activation factor that is itself activated by HP may exist upstream of Mrr in the SOS cascade. As we could not find mutants defective in this putative factor in spite of intensive selection, this factor might be essential to survival under normal-growth conditions. Given the preference of Mrr for a specifically methylated substrate, a still cryptic methylase could be such a factor, because neither *dam, dcm* or *hsdRMS* seem to influence Mrr activity (Waite-Rees et al., 1991; this study).

Although the actual molecular mechanism behind HP-mediated Mrr activation remains to be elucidated, this study provides evidence for a novel pathway that employs a specific type IV restriction endonuclease to shunt the perception of a stress, unrelated to DNA damage, to induction of the SOS response in *E. coli*. Although it could be argued that HP is an unlikely stress to be experienced by *E. coli*, it should be noted that the cellular perception of HP in part can be dissected in cold shock, heat shock and oxidative stress (Welch et al., 1993; Aertsen et al., 2004b; 2005b; Ishii et al., 2005) and thus might well mimic more commonly encountered stresses. Together with the few earlier reports (Taddei et al., 1995; Miller et al., 2004) therefore this study points to the existence of several encoded mechanisms that allow a diverse set of environmental stimuli that do not induce direct DNA damage, to trigger the SOS response. These findings support the idea that bacterial populations use the SOS response as a dedicated mechanism to increase their genetic repertoire and increase their chances of survival in response to various types of stress.

**Experimental procedures**

**Strains and growth conditions**

*Escherichia coli* MG1655 (Blattner et al., 1997) was used as parental (wild-type) strain in this study. MG1655 *lon*::Kn (*lon*:Tn5; Aertsen and Michiels, 2005b), MG1655 *ΔrecA* (*ΔrecA* *srI*:Tn10; Aertsen et al., 2004a) and MG1655 *λ* (*λ*Δ[P3rpoH::*lacZ*]; Aertsen et al., 2004a) were constructed previously. MG1655 *recB*:Kn and MG1655 *recF*:Kn were constructed by P1 transducing *recB*:Tn5 and *recF*:Tn5 from FB21402 and FB20946 (both obtained from Frederick Blattner, Department of Genetics, University of Wisconsin, Madison, WI, USA) respectively. The construction and isolation of MG1655 *lon*::Kn *mrr*::Kn is described below and *mrr*::Kn was P1 transduced to wild-type MG1655 to obtain MG1655 *mrr*::Kn. *E. coli* GM272 (da3-8 cdm-6 hsdS21) was kindly provided by Martin Marinus (Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, USA).

Overnight cultures were obtained by growth in LB (Sambrook et al., 1989) for 21 h at 37°C under well-aerated conditions. Exponential phase cultures were obtained by diluting overnight cultures 1/100 in fresh pre-warmed LB and allowing further incubation until late exponential phase (*OD*$_{600}$ = 0.6) as described earlier (Aertsen et al., 2004a). Antibiotics (Applichem, Darmstadt, Germany) were added when necessary to obtain the following concentrations: 30 µg ml$^{-1}$ chloramphenicol (Cm$^{−}$), 100 µg ml$^{-1}$ ampicillin (Ap$^{125}$) and 20 µg ml$^{-1}$ tetracycline (Tc$^{50}$).

**Treatment with HP, UV and MC**

Treatment of *E. coli* with HP, UV or MC was carried out as described previously (Aertsen et al., 2005a). Briefly, late exponential phase cultures were pelleted by centrifugation (5 min at 6000 g) and resuspended in the same volume of fresh pre-warmed LB. For HP treatment, 500 µl was sealed without air bubbles in a polyethylene bag and pressurized for 15 min in an 8 ml pressure vessel, maintained at 20°C with an external cooling circuit (Resato, Roden, the Netherlands). It should be noted that pressurization caused some adiabatic heating of the sample; however, this was less than 3°C at 100 MPa. For UV treatment, 1 ml portions were poured in a Petri dish and irradiated (0.1 kJ m$^{-2}$) in a UV oven, equipped with five fluorescent lamps of 8 W each, and emitting from 180 to 280 nm with a peak at 254 nm (Bio-Link, Vilber Lourmat, France). UV doses were programmed and are controlled by a radiometer that constantly monitors the UV light emission. The distance between the lamps and the plates was 14 cm. MC treatment was achieved by addition of MC to a final concentration of 2 µg ml$^{-1}$.

After treatment, cultures were maintained at 37°C and used for the measurement of *OD*$_{600}$ and/or *gfp* induction, or for the determination of phage titer and/or viability. For HP or UV cycling treatments, the treated cultures were used to
inoculate (1/100) fresh pre-warmed LB and were regrown to stationary phase. Subsequently, late exponential phase cultures were obtained as described above and used for the following cycle of HP or UV treatment.

**Construction of a random transposon knock-out library and selection of mutations that rescue HP resistance in a lon mutant**

Transposon knock-out mutants of the pressure-sensitive MG1655 _lon::Kn_ strain were constructed using λ, NK1324, carrying a mini-Tn10 transposon with a chloramphenicol resistance gene, following the protocol described by Kleckner _et al._ (1991). Individual insertion mutants were grown in microplate wells and pooled per plate, i.e. 96 independent clones per pool, and stored in 25% glycerol at −80°C. As such, 63 pools were prepared, representing c. 60 000 independent insertion mutants. Each pool was individually screened for rescued (i.e. pressure resistant) insertion mutants by subjecting it to two subsequent HP (100 MPa) cycles. After the second HP cycle, dilutions were plated on LB plates and survival was compared with MG1655 _lon::Kn_ after two HP cycles. One of the pools displayed a c. 10-fold increased survival compared to the _lon_ mutant and to the other pools, and some of the survivors of this pool were purified and retested to confirm their pressure resistance. The chloramphenicol marker of one of these HP resistant insertion mutants was P1 transduced to MG1655 _lon::Kn_ to confirm the linkage of the insertion to rescue of HP resistance. Subsequently, through subcloning and sequencing, the transposance insertion site was mapped to the _mrr_ gene and the allele was designated _mrr::Cm_ (Fig. 5).

**Analysis of SOS induction using transcriptional fusions to gfp**

Several _E. coli_ strains were transformed with an earlier constructed (Aertsen _et al._, 2004a) transcriptional fusion of the promoter of MG1655 _recA_ to _gfp_ or with a transcriptional fusion of the promoter of MG1655 _mrr_ to _gfp_ (pAA820, see construction below). After treatment with HP, UV or MC, 300 μl samples were transferred to microplate wells and placed in a fluorescence reader (Fluoroscan Ascent FL, Thermolabsystems, Brussels, Belgium). Fluorescence at 520 nm was then measured at 30 min intervals with intermittent shaking (every 5 min) at 37°C, using an excitation wavelength of 480 nm. At the same time, OD₆₀₀ was measured and fluorescence was expressed per unit of OD₆₀₀. Alternatively, 3 h after induction, cultures were analysed by flow cytometry analysis using a FACSCalibur apparatus (Becton Dickinson, Erembodegem, Belgium) fitted with an argon laser emitting at 488 nm. Fluorescence data shown are representative results of at least four independent experiments.

**Determination of phage titer**

As described previously (Aertsen _et al._, 2004a; 2005a), 30 μl of chloroform was added to 500 μl portions of a λ lysogenic culture and the mixture was vigorously vortexed. This treatment completely killed the bacterial cells. Subsequently, the mixture was centrifuged (5 min at 24 000 g) and dilutions of the supernatant were then added to 1 ml of stationary phase C600 cells, which were a more efficient plating host than MG1655. Finally 3 ml of TBMM top agar (Kleckner _et al._, 1991) was added to this culture and the mixture was poured on an LB agar plate. After 24 h plaques were counted and the phage titer calculated as plaque-forming units (pfu) per millilitre of the original culture. Induction of phage production was expressed as the log₁₀ of the induction factor (IF), which was obtained by dividing phage titers of treated and untreated samples.

**Determination of viability**

Serial dilutions from treated and untreated samples were plated on LB with a spiral plater (Spiral Systems, OH, USA). Twenty-four hours later, colonies on the plates were counted and reduction factors (RF) were determined as follows:

\[
RF = \frac{\text{cfu/ml (untreated sample)}}{\text{cfu/ml (treated sample)}}
\]

**Construction of plasmids**

The _mrr_ gene together with its own promoter was amplified from MG1655 by PCR (Platinum Pfx DNA polymerase, Invitrogen, Merelbeke, Belgium), using the following primers: 5'-TAGCG GATCCCTGGTTACCAATGCGGCGTA-3' and 5'-CGATAAGCGTTCGTTTGCGGGGTTGAGG-3', providing a BamHI and HindIII recognition site in their respective 5' extensions. The c. 1.5 kb _mrr_ amplicon was subsequently cloned into pACYC184 opened with BamHI and HindIII. The resulting plasmid, pAA810, thus contains the MG1655 _mrr_ gene with its own promoter and now only confers chloramphenicol resistance. In the same way, _mrrII_ from MG1655 _lon::Kn_ and _mrrVII_ from MG1655 _lon::Kn_ (Fig. 5) were cloned into pACYC184, creating pAA811 and pAA812 respectively. pAA811 and pAA812 both produce a non-functional Mrr protein, and are used as control plasmids. Plasmids were transformed to various strains by electroporation.

An _mrr_ transcriptional promoter fusion to _gfp_ was created by amplifying a c. 0.5 kb fragment directly upstream the _mrr_ start codon, using the following primers: 5'-TAGCGGATCC CCTGGTTACCAATGCGGCGTA-3' and 5'-CGATCTAGATTTGTTGCCAGATAACGCAGAC-3', providing a BamHI and XbaI recognition site respectively. The _mrr_ promoter amplicon was subsequently cloned into pPPV25 (Valdivia and Falkow, 1996), containing a promoterless _gfp_ gene, and cut with BamHI and XbaI. The resulting plasmid, pAA820, contains the _mrr_ promoter directly upstream of _gfp_ and was transformed to MG1655.

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