

Protection of the DNA during the exposure of *Escherichia coli* cells to a toxic metabolite: the role of the KefB and KefC potassium channels

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Summary

The effect of the toxic metabolite methylglyoxal on the DNA of *Escherichia coli* cells has been investigated. Exposure of *E. coli* cells to methylglyoxal reduces the transformability of plasmid DNA and results in the degradation of genomic DNA. The activity of the KefB and KefC potassium channels protects *E. coli* cells against methylglyoxal and limits the amount of DNA damage. In mutants lacking KefB and KefC, methylglyoxal-induced DNA damage was reduced by incubation with a weak acid that lowers the pH to the same extent as through KefB and KefC activation. This provides evidence that acidification of the cytoplasm protects *E. coli* DNA against methylglyoxal. By the analysis of cells lacking UvrA, we demonstrate that this repair protein is required for the degradation of the DNA upon methylglyoxal exposure. However, protection by KefB and KefC occurred independently of UvrA. Although we present evidence that exposure of *E. coli* cells to methylglyoxal results in DNA degradation, our results suggest this event is not essential for methylglyoxal-induced death. The implications of these findings will be discussed.

Introduction

Bacterial cells are subject to stress both generated within and encountered in their environment. One such stress

arises from the production of methylglyoxal, a toxic electrophile produced in all cells by several different mechanisms (Murata *et al.*, 1989). In *Escherichia coli* cells, the major route of methylglyoxal production is from the glycolytic intermediate dihydroxyacetone phosphate (DHAP) by the action of methylglyoxal synthase (MGS) (Hopper and Cooper, 1971; Totemeyer *et al.*, 1998). MGS converts DHAP to methylglyoxal with the release of inorganic phosphate. Cells of *E. coli* synthesize millimolar quantities of methylglyoxal when grown on a 'poor' carbon source such as D-xylose in the presence of cAMP (Ackerman *et al.*, 1974; Ferguson *et al.*, 1993, 1996). The accumulation of methylglyoxal in *E. coli* cells results in growth inhibition, and at higher concentrations (> 0.4 mM) death occurs (Ferguson *et al.*, 1995, 1996, 1998; Ferguson and Booth, 1998; Ferguson, 1999). However, the precise concentrations at which these events occur are dependent upon multiple cellular and environmental factors (Ferguson *et al.*, 1998; Ferguson, 1999). The mechanism(s) by which methylglyoxal either inhibits growth or results in death of the bacterial cell is poorly understood and may be different depending upon precise conditions. It is thought that the toxicity of methylglyoxal to cells arises from its ability to interact with the nucleophilic centres of macromolecules (Ferguson *et al.*, 1995, 1998; Ferguson, 1999). These interactions would clearly have profound effects on the functioning of macromolecules. In particular, if methylglyoxal damages the DNA of the cell then this would have to be repaired to ensure the long-term survival of the bacterium. It has been found that methylglyoxal reacts *in vitro* most readily with the base guanine and to a lesser extent with adenine and cytosine (Papoulis *et al.*, 1995). The reaction of methylglyoxal with guanine generates N²-(1-carboxyethyl)guanine (CEG) as the major product. As yet, CEG has not been detected *in vivo*, although methylglyoxal is mutagenic to the DNA of *Salmonella typhimurium* cells (Marnett *et al.*, 1985).

To ensure their survival in the presence of electrophiles such as methylglyoxal, bacteria have multiple protective mechanisms. In *E. coli* cells, the tripeptide glutathione plays a central role in protection (Apontowiel and Berends, 1975; Ferguson and Booth, 1998). Glutathione reacts spontaneously with methylglyoxal to generate hemithiolacetal (HTA) and this is then metabolized to D-lactate by the

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consecutive actions of glyoxalase I and II (Cooper, 1984; MacLean *et al.*, 1998). During the conversion of HTA to D-lactate, S-lactoylglutathione (SLG) is produced. The production of SLG activates the KefB and KefC potassium channels, resulting in the rapid release of intracellular potassium (Ferguson *et al.*, 1993; MacLean *et al.*, 1998). The loss of potassium from the cell is compensated by the influx of protons, resulting in acidification of the cytoplasm (Bakker and Mangerich, 1982; Ferguson *et al.*, 1995, 1996; Ferguson and Booth, 1998). This rapid decrease in the intracellular pH (pHi) protects the *E. coli* cell against methylglyoxal-induced death (Ferguson *et al.*, 1995, 1996; Ferguson and Booth, 1998). The protection afforded by acidification of the cytoplasm was separate from detoxification because it did not alter the rate at which methylglyoxal was metabolized (Ferguson *et al.*, 1995; Ferguson and Booth, 1998). The pHi of cells had to fall beneath 7.4 within 30 min of methylglyoxal addition to achieve full protection (Ferguson *et al.*, 1995, 1996). If the pHi decrease was delayed, then only a proportion of cells could be rescued. Protection by acidification of the cytoplasm was unaffected by incubation of cells in the presence of the protein synthesis inhibitor chloramphenicol, providing evidence that new protein synthesis was not required (Ferguson *et al.*, 1995). These findings led to the proposal that acidification of the cytoplasm was required to protect *E. coli* cells against methylglyoxal-induced damage, either directly or through the activation of pre-existing repair systems (Ferguson *et al.*, 1995, 1998; Ferguson, 1999). In this paper, we set out to investigate the effect of methylglyoxal on the DNA of *E. coli* cells. We demonstrate that exposure of *E. coli* cells to methylglyoxal leads to DNA damage and that the activity of the KefB and KefC systems limit this damage. We also show that UvrA is responsible for methylglyoxal-induced degradation of genomic DNA, but protection by KefB and KefC is independent of UvrA. In addition, we provide evidence that DNA degradation is not essential for methylglyoxal-induced cell death.

Results

The activity of the KefB and KefC channels protects plasmid DNA on exposure of E. coli cells to methylglyoxal

To investigate the effect of methylglyoxal on the DNA of *E. coli* cells, we sought to use a plasmid as a reporter of DNA damage. We proposed that if methylglyoxal was damaging the DNA *in vivo* then transformation of a plasmid into another *E. coli* strain should lead to a reduction in the transformation efficiency. Exponential phase cells of MJF274 (KefB⁺, KefC⁺) and MJF276 (KefB⁻, KefC⁻) carrying pHSG575 (Takeshita *et al.*, 1987) were exposed to a range of methylglyoxal concentrations (0–0.6 mM) for

3.5 h in 0.2 mM K (K_{0.2}) medium and the viability determined (Fig. 1A). It has been shown previously that 0.2 mM potassium allows the growth of our *E. coli* strains without affecting the activity of the KefB and KefC systems (Ferguson *et al.*, 1993, 1995). The viability was determined (Fig. 1A), the plasmids isolated and then 200 ng of pHSG575 DNA transformed into NM522 cells (Fig. 1B). Cells possessing the KefB and KefC channels were better protected against methylglyoxal-induced death than cells lacking these systems (Fig. 1A). Likewise, the transformation efficiency of pHSG575 extracted from cells possessing KefB and KefC was greater than from cells deficient in these systems after exposure to all the concentrations of methylglyoxal tested (Fig. 1B). The KefB and KefC channels appeared to provide a high level of protection to pHSG575 when cells were exposed up to 0.5 mM methylglyoxal

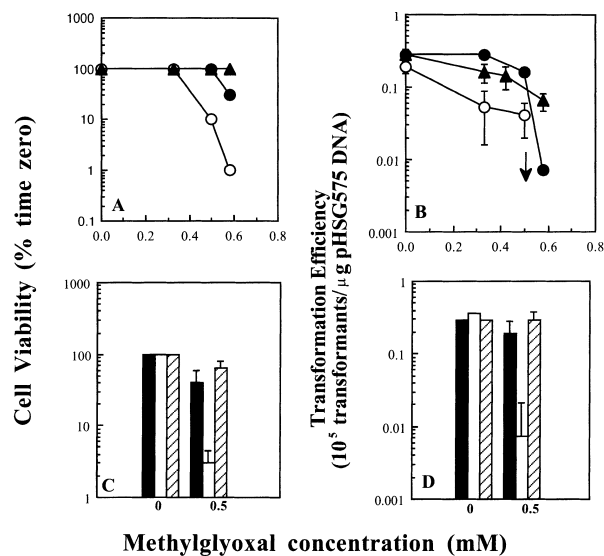


Fig. 1. Activation of the KefB and KefC systems protects pHSG575 DNA on exposure of *E. coli* cells to methylglyoxal. Cell viability and transformation efficiencies were determined exactly as described in *Experimental procedures*.

A. Exponential phase cells of MJF274 (KefB⁺, KefC⁺) (●), MJF276 (KefB⁻, KefC⁻) (○) and MJF276 treated with 25 mM sodium acetate at time 0 (▲), carrying pHSG575, were exposed to a range of methylglyoxal concentrations for 3.5 h in K_{0.2} medium and the viability determined.

B. The plasmids were then isolated, transformed into NM522 cells and the transformants selected on plates supplemented with 10 μg ml⁻¹ chloramphenicol.

C. Exponential phase cells of MJF274 (KefB⁺, KefC⁺) in K_{0.2} medium (filled bars), K₁₀ medium (open bars) and K₁₀ medium supplemented with 25 mM sodium acetate at time 0 (diagonally shaded bars), carrying pHSG575, were exposed to 0 and 0.5 mM methylglyoxal for 3 h and the viability determined.

D. The plasmids were then isolated, transformed into NM522 cells and transformants selected on plates supplemented with 10 μg ml⁻¹ chloramphenicol.

The arrow represents no transformants after the treatment of cells with higher concentrations of methylglyoxal. Where shown, the error bars represent the standard deviation from the mean for one experiment.

because the transformation efficiency was not substantially reduced. In contrast, in the absence of KefB and KefC, the transformation efficiency of pHSG575 was severely affected even at non-lethal concentrations of methylglyoxal. The KefB and KefC channels were also found to protect pBR328 DNA against methylglyoxal-induced damage (data not shown). These data provide evidence that the KefB and KefC channels protect plasmid DNA against methylglyoxal-induced damage.

We have shown previously that the activity of the KefB and KefC channels in the presence of methylglyoxal could be inhibited by increasing the potassium concentration of the medium from 0.2 to 10 mM (K_{10} medium; Ferguson *et al.*, 1993; Ferguson and Booth, 1998). To confirm that the activity of KefB and KefC was essential to protect plasmid DNA against methylglyoxal, exponential phase cells of MJF274 carrying pHSG575 were exposed to either 0 or 0.5 mM methylglyoxal for 3 h in either $K_{0.2}$ or K_{10} medium. The viability was determined (Fig. 1C), the plasmids isolated and then 200 ng pHSG575 DNA transformed into NM522 cells (Fig. 1D). Consistent with previous findings, supplementation of the medium with 10 mM potassium sensitized cells of MJF274 upon exposure to methylglyoxal (Fig. 1C). In addition, the transformation efficiency of pHSG575 was dramatically reduced under these conditions (Fig. 1D). These data provide evidence that the activity of the KefB and KefC channels protects plasmid DNA against methylglyoxal.

Acidification of the cytoplasm protects plasmid DNA after methylglyoxal addition

In an earlier study, we have shown that the rapid lowering of the pHi, which occurs as a consequence of the activation of KefB and KefC, protects cells of *E. coli* on exposure to electrophiles (Ferguson *et al.*, 1995, 1996, 1997; Ferguson and Booth, 1998). This was established by manipulating the pHi of cells lacking KefB and KefC with weak acids such as acetate. The addition of 25 mM sodium acetate was found to decrease the pHi and protect fully cells of either MJF274 (KefB⁺, KefC⁺) in K_{10} medium or MJF276 (KefB⁻, KefC⁻) in $K_{0.2}$ medium during exposure to 0.5 mM methylglyoxal (Ferguson *et al.*, 1995). This was also found to be the case when the same strains carried pHSG575 (Fig. 1A and C respectively). Treatment of cells of either MJF274 in K_{10} or MJF276 in $K_{0.2}$ with 25 mM sodium acetate also prevented the dramatic decrease in the transformation efficiency of pHSG575 after exposure of cells with methylglyoxal (Fig. 1B and D respectively). These data provided evidence that acidification of the cytoplasm could protect both *E. coli* cells and plasmid DNA against methylglyoxal to a similar extent as through the activation of the KefB and KefC channels.

The KefB and KefC channels reduce the destruction of genomic DNA upon the exposure of *E. coli* cells to methylglyoxal

Having determined that the KefB and KefC channels protected plasmid DNA against methylglyoxal-induced damage, we sought to determine the effect of these systems on the genomic DNA of *E. coli* cells. Exponential phase cells of MJF274 (KefB⁺, KefC⁺) and MJF276 (KefB⁻, KefC⁻) were exposed to a range of methylglyoxal concentrations (0–0.7 mM) for 3 h in $K_{0.2}$ medium, the genomic DNA isolated and then analysed on an agarose gel (Fig. 2A). Treatment of cells with increasing concentrations of methylglyoxal resulted in progressively less

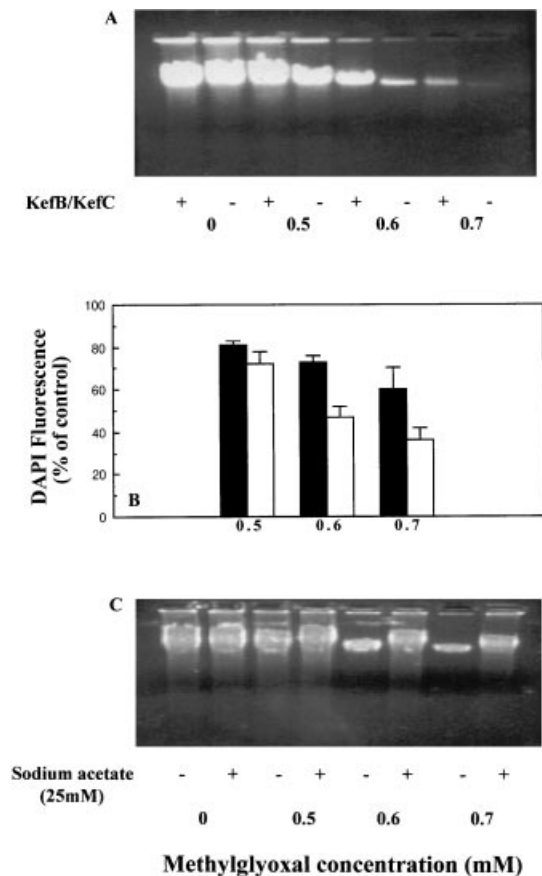


Fig. 2. The KefB and KefC systems reduce the loss of genomic DNA after treatment of *E. coli* cells with methylglyoxal. The genomic DNA was isolated and the DAPI staining was conducted exactly as described in *Experimental procedures*. A. Exponential phase cells of MJF274 (KefB⁺, KefC⁺) and MJF276 (KefB⁻, KefC⁻) in $K_{0.2}$ medium were exposed to a range of methylglyoxal concentrations for 3 h and then the genomic DNA was isolated and analysed. B. Cells of MJF274 (filled bars) and MJF276 (open bars) were treated as in A and then stained with 4,6-diamidino-2-phenylindole.2HCl (DAPI). The error bars represent the standard deviation from the mean for one experiment. C. Same as in A, except cells of MJF276 were treated with or without 25 mM sodium acetate at time 0.

genomic DNA being extracted. The genomic DNA of cells lacking KefB and KefC was more severely affected at all the concentrations of methylglyoxal tested than DNA extracted from cells possessing KefB and KefC. However, in the presence of 0.7 mM methylglyoxal, the concentration of genomic DNA extracted from both MJF274 and MJF276 cells was greatly reduced. The reduction in the level of genomic DNA was not due to the lysis of dead cells as the optical density of the culture was unaltered in the presence of methylglyoxal (data not shown). In addition, in cells in which the DNA was labelled with methyl- ^{3}H -thymidine there was no release of high molecular weight material into the medium upon methylglyoxal addition (data not shown). These data suggest that methylglyoxal results in the degradation of the genomic DNA of *E. coli* cells and that the KefB and KefC channels appear to minimize this effect. However, it was also possible that methylglyoxal altered the genomic DNA such that it was lost during the isolation procedure and this effect could be greatest in cells lacking KefB and KefC.

To confirm that methylglyoxal resulted in the destruction of the genomic DNA, an alternative procedure was followed that did not rely on the isolation of DNA. Exponential phase cells of MJF274 and MJF276 were treated with methylglyoxal and then the cells embedded in agarose. The cells were lysed within the agarose, treated to remove proteins and RNA such that only the genomic DNA remained and this was then analysed by pulsed-field gel electrophoresis. Consistent with our earlier observations (Fig. 2A), treatment of cells with methylglyoxal led to a decrease in the genomic DNA, and this was greatest for cells lacking the KefB and KefC channels (data not shown). Further evidence supporting the destruction of the genomic DNA upon methylglyoxal treatment was obtained by the use of 4',6'-diamidino-2-phenylindole 2HCl (DAPI) staining. DAPI is a specific dye that forms a fluorescent complex upon binding DNA. However, DAPI has the advantage that it can be used to measure DNA in fixed *E. coli* cells. Exponential phase cells of MJF274 and MJF276 were treated with 0–0.7 mM methylglyoxal for 3 h and then the effect on the genomic DNA was analysed using DAPI staining (Fig. 2B). Treatment of cells with methylglyoxal resulted in a decrease in the level of DAPI fluorescence, suggesting that there was a reduction in the amount of genomic DNA. The decrease in fluorescence was greater for cells lacking KefB and KefC than for cells possessing these systems. These data further support the hypothesis that the addition of methylglyoxal to *E. coli* cells results in the degradation of the genomic DNA and that the KefB and KefC channels reduce the amount of damage. In contrast to our findings with the genomic DNA, methylglyoxal treatment of *E. coli* cells did not significantly reduce the level of plasmid DNA extracted. This was determined by analysing the extracted plasmids using agarose gel electrophoresis

and by measuring the absorbance at 260 and 280 nm of the plasmid preparations (data not shown).

To investigate whether the protection of the genomic DNA by the KefB and KefC channels after methylglyoxal treatment was due to acidification of the cytoplasm, 25 mM sodium acetate was added to cells of MJF276 (KefB⁻, KefC⁻) immediately before methylglyoxal exposure. Consistent with the ability of acetate to protect cells against methylglyoxal (Fig. 1A and C respectively), the addition of sodium acetate also reduced the destruction of genomic DNA after exposure of MJF276 cells to 0–0.7 mM methylglyoxal for 3 h (Fig. 2C). The reduction in destruction of the genomic DNA by acetate treatment of MJF276 cells followed a similar trend to that observed by activation of the KefB and KefC systems (Fig. 2C and A respectively). This provided evidence that acidification of the cytoplasm was responsible for KefB/KefC-mediated protection of genomic DNA against methylglyoxal.

It has been shown previously that *E. coli* cells continuously produce methylglyoxal when grown on a 'poor' carbon source such as D-xylose in the presence of cAMP (Ackerman *et al.*, 1974; Ferguson *et al.*, 1993, 1996). The continuous production of methylglyoxal by *E. coli* cells results in cell death and genomic DNA destruction as the concentration of this toxic electrophile accumulates (Fig. 3A and B respectively). However, consistent with the data obtained for the externally added methylglyoxal, the KefB and KefC channels reduced the amount of cell death and DNA destruction in the presence of endogenously produced methylglyoxal.

The genomic DNA is degraded into low molecular weight material upon methylglyoxal exposure in a UvrA-dependent process

It was observed earlier that the exposure of cells of MJF274 (KefB⁺, KefC⁺) and MJF276 (KefB⁻, KefC⁻) to 0.7 mM methylglyoxal for 3 h resulted in the substantial loss of genomic DNA from both strains (Fig. 2A and B). Hence, we sought to determine more precisely the timing of the destruction of the genomic DNA. Exponential phase cells of MJF274 and MJF276 in K_{0.2} medium were exposed to 0.7 mM methylglyoxal for 0–4 h. The cells were harvested and the genomic DNA isolated and analysed on an agarose gel (Fig. 4A). These data showed that the genomic DNA progressively decreased throughout the time course of exposure to methylglyoxal. However, the destruction of the DNA from cells lacking KefB and KefC was more rapid than for cells possessing these systems and it started to occur after exposure to 0.7 mM methylglyoxal for 1 h. Exposure of MJF276 cells to 0.7 mM methylglyoxal for less than 1 h did not result in any significant degradation of the DNA (data not shown). These data provide further evidence that the KefB and KefC channels delay the

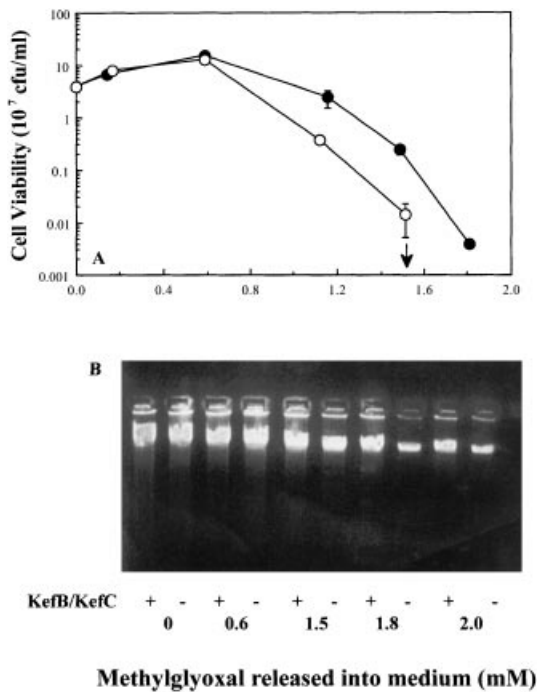


Fig. 3. The KefB and KefC systems delay the destruction of genomic DNA under conditions resulting in high levels of methylglyoxal production in *E. coli* cells. The methylglyoxal assay, cell viability and isolation of genomic DNA were performed exactly as described in *Experimental procedures*.

A. Exponential phase cells of MJF274 (KefB⁺, KefC⁺) (●) and MJF276 (KefB⁻, KefC⁻) (○) in K_{0.2} medium with xylose as the sole carbon source were treated with 2 mM cAMP at time 0. The cell viability was determined and plotted against the concentration of methylglyoxal released into the medium. Where shown, the error bars represent the standard deviation from the mean for one experiment and the arrow represents no viable cells by the next time point.

B. Same as in A, except that the genomic DNA was isolated.

destruction of the DNA in the presence of high concentrations of methylglyoxal.

Having determined that methylglyoxal resulted in the destruction of genomic DNA, we sought to investigate the fate of the DNA. Analysis of the genomic DNA suggested that it was not being broken into fragments in the presence of methylglyoxal, but rather completely degraded, because no smear was detected (Figs 2A and C, 3B and 4A). The DNA fragments were not being lost during the isolation procedure because they were also not observed on the pulsed-field gels (data not shown). To investigate the fate of the DNA further, exponential phase cells of MJF276 (KefB⁻, KefC⁻) were prepared in the presence of methyl-[³H]-thymidine to label the DNA. The cells were then exposed to 0.7 mM methylglyoxal for 0–3 h and the fate of ³H label determined (Fig. 4B and C). The level of ³H label present in the low molecular weight (< 3000) cellular fraction increased with lengthening exposure time to methylglyoxal (Fig. 4B). In addition, the ³H label in the

medium also increased upon exposure to methylglyoxal (Fig. 4C). This increase was not due to cell lysis as no radioactivity was present in the medium fraction with a molecular weight > 3000 (data not shown). These data suggest that in the presence of methylglyoxal the DNA of *E. coli* cells is rapidly degraded into low molecular weight material.

The data presented in this paper so far provide evidence that the accumulation of methylglyoxal results in the destruction of the genomic DNA. However, the mechanism by which the DNA was degraded was unknown. The destruction of the DNA has also been observed previously after *E. coli* cells were exposed to high doses of UV irradiation (Bonura and Smith, 1975). It was shown that the

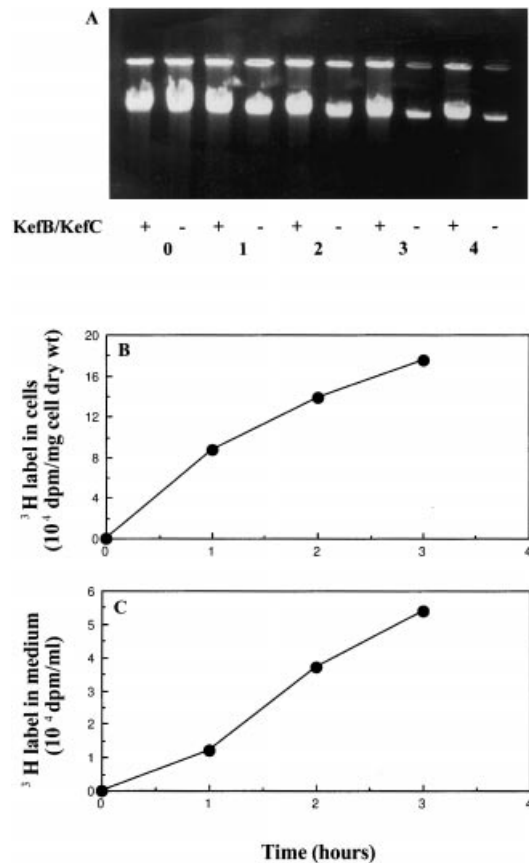


Fig. 4. Methylglyoxal results in the destruction of the DNA into low molecular weight material. The isolation of the genomic DNA, the ³H labelling of the DNA and the analysis of the ³H label in the cells and medium were conducted exactly as described in *Experimental procedures*.

A. Exponential phase cells of MJF274 (KefB⁺, KefC⁺) and MJF276 (KefB⁻, KefC⁻) were exposed to 0.7 mM methylglyoxal in K_{0.2} medium for the defined length of time and then the genomic DNA isolated.

B. Exponential phase cells of MJF276, previously grown in the presence of methyl-[³H]-thymidine, were treated as described in A, then the amount of ³H label in the low molecular weight (< 3000) cellular fraction determined.

C. As B, except the ³H label in the medium was determined.

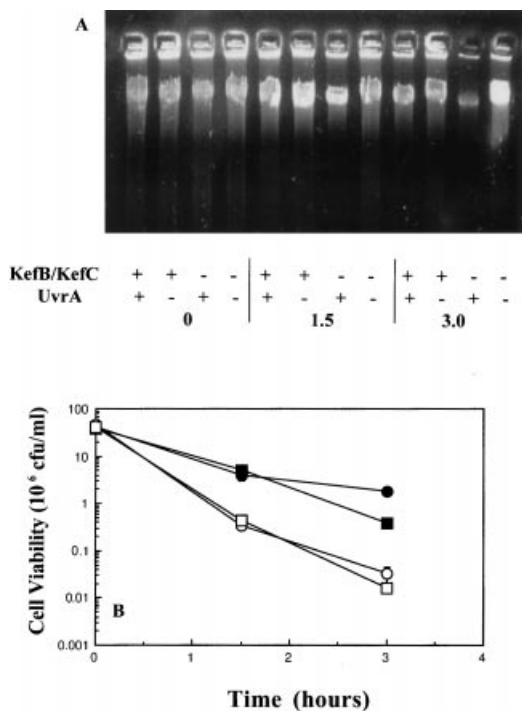


Fig. 5. Degradation of the genomic DNA upon exposure to methylglyoxal is dependent upon UvrA. The isolation of genomic DNA and cell viability was determined exactly as described in *Experimental procedures*, except that cells were grown in the dark because of the sensitivity of the UvrA-deficient mutant to light. A. Exponential phase cells of MJF274 (KefB⁺, KefC⁺), MJF489 (MJF274, UvrA⁻), MJF364 (KefB⁻, KefC⁻) and MJF490 (MJF364, UvrA⁻) were treated with or without 0.7 mM methylglyoxal as defined, and then the genomic DNA was isolated. B. As A, except that the cell viability was determined. MJF274 (●); MJF489 (■); MJF364 (○) and MJF490 (□). Where shown, the error bars represent the standard deviation from the mean for one experiment.

degradation of the DNA by UV irradiation was dependent upon the presence of UvrA, a subunit of the Uvr(A)BC excinuclease. Hence, we investigated whether this DNA repair enzyme could also be involved in the degradation of the DNA upon the exposure of *E. coli* cells to methylglyoxal. To test this possibility, exponential phase cells of MJF274 (KefB⁺, KefC⁺, UvrA⁺), MJF489 (KefB⁺, KefC⁺, UvrA⁻), MJF364 (KefB⁻, KefC⁻, UvrA⁺) and MJF490 (KefB⁻, KefC⁻, UvrA⁻) in K_{0.2} medium were exposed to 0.7 mM methylglyoxal for 0–3 h and the genomic DNA analysed and cell viability determined (Fig. 5A and B respectively). Consistent with our proposal, in the absence of UvrA, there was no degradation of the DNA in the presence of methylglyoxal, independent of the activity of the KefB and KefC systems (Fig. 5A). This suggested that the Uvr(A)BC excinuclease played an essential role in methylglyoxal-induced degradation of the DNA. However, although there was no degradation of the DNA in cells lacking UvrA upon exposure to methylglyoxal, these cells still lost viability to

a similar extent as their UvrA⁺ counterparts (Fig. 5B). These data suggest that protection by the KefB and KefC channels was independent of UvrA and that the degradation of the DNA was not essential for methylglyoxal-induced cell death. It should be noted that after treatment with methylglyoxal there was always some genomic DNA remaining (Figs 2A and C, 3B, 4A and 5A). One possibility was that this remaining DNA was inaccessible to enzyme(s) involved in the degradation. However, the remaining DNA from MJF274 and MJF276 cells could be digested with the restriction enzyme *Not*I before analysis by pulsed-field gel electrophoresis (data not shown). This finding suggests that the residual genomic DNA was not inaccessible to enzymes.

Discussion

The data presented provide evidence that methylglyoxal results in DNA damage *in vivo* and that the activity of the KefB and KefC channels reduces the level of this damage. The transformability of plasmid DNA was substantially reduced by treatment of *E. coli* cells with increasing concentrations of methylglyoxal. This could have occurred if either the general integrity of the plasmid was affected or if there were mutations in the antibiotic resistance genes or origin of replication. It is likely that methylglyoxal can interact directly with plasmid DNA *in vivo* because it can interact with bases *in vitro* under physiological conditions to form CEG as the major product (Papoulis *et al.*, 1995). The direct interaction of [¹⁴C]-methylglyoxal with DNA has also been observed in eukaryotic cells (Kang *et al.*, 1996). However, it is possible that methylglyoxal can also damage plasmid DNA indirectly. For example, *in vitro* methylglyoxal can react with amino acids to generate superoxide and this would result in oxidative damage to DNA if produced *in vivo* (Yim *et al.*, 1995). It has been shown previously that the SoxR protein is activated by internally generated superoxide, which then activates transcription of the *soxS* gene (Nunoshiba *et al.*, 1992). However, there was no induction of *soxS::lacZ* during the exposure of *E. coli* cells to methylglyoxal, suggesting that large quantities of superoxide are not accumulating *in vivo* (C. McIntosh and G. P. Ferguson, unpublished).

Methylglyoxal results in the degradation of genomic DNA within *E. coli* cells into low molecular weight material (< 3000). Therefore, if the DNA of *E. coli* cells remained double stranded after methylglyoxal exposure, the largest fragment after degradation would be less than 5 bp. It has been shown previously that treatment of human leukaemia 60 (HL60) cells with methylglyoxal also results in degradation of the DNA (Kang *et al.*, 1996). However, the degradation of DNA in HL60 cells produced a DNA ladder on agarose gels, characteristic of apoptosis. This laddering of the DNA did not occur with *E. coli* cells and is probably

owing to the differences in the organization of the genome in eukaryotic and prokaryotic cells. In HL60 cells, the degradation of the DNA was preceded by the formation of methylglyoxal–DNA adducts (Kang *et al.*, 1996). It was proposed that the formation of these adducts activated cellular endonucleases, resulting in degradation of the DNA. Previous work has shown that double-stranded breaks (DSBs) occur in the DNA after exposure of *E. coli* cells to high doses of UV radiation (Bonura and Smith, 1975). The UvrA protein played an essential role in the formation of these DSBs because they were not detected in UvrA-deficient mutants. The combined activities of the UvrA, UvrB and UvrC proteins form the (A)BC excinuclease, which repairs a wide range of DNA lesions with little structural similarities (Sancar, 1996). At low doses of UV, the Uvr(A)BC enzyme excises a 12- to 13-nucleotide oligomer around the damaged lesions, leaving gaps that are then repaired by DNA polymerase I and ligase. However, at higher doses of UV, there is a greater frequency of gaps, and DSBs are created when two gaps overlap. We have shown that the UvrA protein is essential for the degradation of *E. coli* DNA in the presence of methylglyoxal as in the absence of this protein no loss of genomic DNA was observed. In contrast, plasmid DNA, although damaged, was not degraded in the presence of methylglyoxal. A possible explanation is that the methylglyoxal-induced lesions in the plasmids were far enough apart that DSBs were not created.

We present data that the exposure of *E. coli* cells to methylglyoxal results in DNA damage and degradation. However, the question arises as to whether this DNA damage and/or degradation is responsible for methylglyoxal-induced cell death. The degradation of the DNA did not appear essential for methylglyoxal-induced cell death because no loss of the DNA occurred in the UvrA-deficient cells, although these cells were equally susceptible to methylglyoxal compared with their UvrA⁺ counterparts. This suggests that the methylglyoxal-induced DNA lesions could be sufficient to result in cell death. However, we cannot rule out other factors being involved. For example, it has been shown previously that methylglyoxal can react *in vitro* with the amino acids lysine, arginine and cysteine under physiological conditions and can also inhibit the activity of some of the glycolytic enzymes (Leoncini *et al.*, 1980; Lo *et al.*, 1994). However, the concentrations required to inhibit enzymes were several-fold higher than in our experiments and, hence, it is unlikely that this would be responsible for methylglyoxal-induced cell death (Leoncini *et al.*, 1980).

The activity of the KefB and KefC channels was found to reduce methylglyoxal-induced plasmid damage and degradation of the genomic DNA. Treatment of cells with acetate, to lower the pHi by the same extent as by the activation of KefB and KefC, also reduced DNA damage by

methylglyoxal. These data add support to our previous findings and suggest that acidification of the cytoplasm is responsible for KefB/KefC-mediated protection against methylglyoxal (Ferguson *et al.*, 1995; Ferguson and Booth, 1998). The mechanism by which acidification of the cytoplasm reduces methylglyoxal-induced DNA damage requires investigation. We have shown that acidification of the cytoplasm does not protect *E. coli* cells by reducing the activity of the Uvr(A)BC excinuclease because protection by KefB and KefC occurred in cells lacking UvrA. However, in cells possessing UvrA, the KefB and KefC channels could reduce the amount of DNA degradation compared with cells lacking these systems. This finding suggests that there must be less methylglyoxal-induced DNA lesions in cells possessing KefB and KefC. The ability of KefB and KefC to reduce methylglyoxal-induced plasmid damage also added support to this proposal. It is possible that changes in the pHi could influence the reactivity of methylglyoxal with DNA. Alternatively, the changes may affect the activity of a pre-existing DNA repair enzyme. It has been shown previously *in vitro* that the ability of the *E. coli* 3-methyladenine DNA glycosylase II (AlkA) to excise guanine residues is increased 10-fold by lowering the pH from 7.9 to 6.5 (Berdal *et al.*, 1998). Methylglyoxal has been found to react most readily with guanine residues *in vitro* (Papoulis *et al.*, 1995), and hence acidification of the cytoplasm could protect *E. coli* cells against methylglyoxal by activating AlkA and repairing damaged DNA.

In an earlier study, it was shown that acidification of the cytoplasm, by weak acid addition, protects cells of *E. coli* against another electrophile, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Oktyabrsky *et al.*, 1993). It was found that reducing the intracellular pH to levels similar to our experiments caused a decrease in the non-protein thiol pool by ≈25% within 15 min. In *E. coli* cells, glutathione is the major non-protein thiol present in the cytoplasm and MNNG is activated to a mutagen by reaction with glutathione (Fahey *et al.*, 1978; Kumaresan *et al.*, 1995). Therefore, a reduction in the level of glutathione by acidification of the cytoplasm would result in less MNNG becoming mutagenic. In contrast, glutathione detoxifies and protects *E. coli* cells against methylglyoxal (Apontoweil and Berends, 1975; Ferguson and Booth, 1998). Thus, a reduction in the glutathione level by acidification of the cytoplasm would not lead to protection against methylglyoxal. In addition, we have shown previously that acidification-mediated protection of *E. coli* cells against methylglyoxal can occur in a glutathione-deficient mutant (Ferguson and Booth, 1998). This provides evidence that changes in the level of glutathione are not responsible for acidification-mediated protection against methylglyoxal.

In conclusion, we have demonstrated that upon exposure to the toxic metabolite methylglyoxal the DNA of *E. coli* cells is damaged and degraded in a UvrA-dependent

manner. It is interesting to speculate whether the degradation of genomic DNA by the Uvr(A)BC excinuclease is either an unfortunate consequence of excessive DNA repair activity or whether it offers some benefit to cells in the population. For example, the release of nucleotides into the environment could be utilized by surviving bacteria. It is also of interest that the *E. coli* cell provides the 'tools' for its own demise; methylglyoxal is a naturally occurring metabolite and UvrA forms part of a DNA repair system. Hence, it may be possible to target these systems in the design of novel antibacterial agents and essentially get the bacterial cell to commit suicide.

Experimental procedures

Bacterial strains and plasmids

The *E. coli* strains used in this study were: MJF274 ($F^- \Delta kdp$ *thi rha lacI lacZ kup*), MJF276 (MJF274, *kefB kefC::Tn10*), MJF364 (MJF274, *kefB yabF-kefC::kan*), MJF489 (MJF274, *uvrA::Tn10*), MJF490 (MJF364, *uvrA::Tn10*) and NM522 [*supE thi Δ(lac-proAB) Δ(mcrB-hsdSM)5(r_k⁻m_k⁻)* (*F' proAB lacI^qΔM15*)]. For the plasmid test, pBR328 (nbl Gene Sciences) and pHSG575 (Takeshita *et al.*, 1987) were used. pBR328 is 4.9 kb, has a copy number of ≈ 30 –40 and confers resistance to ampicillin, tetracycline and chloramphenicol. pHSG575 is 3.6 kb, has a copy number of 3–5 and confers resistance to chloramphenicol.

Growth media

All cells were grown as stated in the text. The minimal medium used was K_x (where x is the concentration of potassium in mM; Epstein and Kim, 1971) and was supplemented with 0.2% glucose as the carbon source, except for the endogenous production of methylglyoxal. When the cells were transformed either with pBR328 or pHSG575, the medium was also supplemented with either $25 \mu\text{g ml}^{-1}$ ampicillin or $10 \mu\text{g ml}^{-1}$ chloramphenicol respectively. K_0 buffer was used to wash cells and lacked all growth supplements. With the exception of the preparation of competent cells, exponential phase cells were prepared as follows: an overnight culture in K_x medium was diluted 15-fold into fresh medium and grown to an OD_{650} 0.4. The culture was then diluted 10-fold into fresh medium and methylglyoxal was added to the concentration as defined in the text. Cell viability was determined exactly as has been described previously (Ferguson *et al.*, 1993, 1995). Recovery of cells was conducted in K_0 buffer and then they were spotted onto K_{10} plates. For the induction of endogenous methylglyoxal, cells were grown in medium containing 0.2% xylose as the sole carbon source. Methylglyoxal was induced by the addition of 2 mM cAMP exactly as has been described previously (Ackerman *et al.*, 1974; Ferguson *et al.*, 1993). The concentration of methylglyoxal in the supernatant was determined by reaction with 2,4-dinitrophenylhydrazine as has been described previously (Ferguson *et al.*, 1995). For the competent cells, an overnight culture in SOB medium (Gibco BRL) was diluted 50-fold into fresh medium and grown to an OD_{650} 0.6. The competent

cells were then prepared following a standard method and stored at -80°C until required (Lee and Cerami, 1987).

Plasmid test

Exponential phase cells transformed with pBR328 were exposed to methylglyoxal (as defined), diluted to an OD_{650} 0.05 in K_0 buffer and then 90 ml was centrifuged ($4500 \times g$, 15 min). The culture was divided into two and then pBR328 isolated immediately using spin columns (Qiagen) according to the standard procedure. pBR328 was eluted from the column by $50 \mu\text{l}$ TE (pH 8.0) and then the two preparations combined. Because of the low copy number of pHSG575 (Takeshita *et al.*, 1987), a greater volume of culture was required to obtain sufficient quantities of plasmid. After treatment with methylglyoxal of cells transformed with pHSG575, a volume of culture, equivalent to 180 ml OD_{650} 0.05, was filtered (Whatman, $0.45 \mu\text{m}$) and then the cells were resuspended in 50 ml K_0 buffer. The procedure was then followed exactly as above. The plasmid preparations were quantified both by measuring the absorbance at 260 and 280 nm using the Pharmacia Biotechnologies DNA calculator and by agarose gel electrophoresis.

The effect of methylglyoxal treatment on the ability of plasmid DNA to transform NM522 cells was conducted using a modification of a previously described procedure (Lee and Cerami, 1987). NM522 competent cells (0.2 ml) were thawed on ice and then transferred to a 5 ml polypropylene tube. Plasmid DNA (200 ng) was added, the tube mixed gently and stored on ice for 1 h. The cells were heat shocked (42°C , 2 min), placed on ice for a further 5 min and then 0.8 ml of SOC medium (Gibco BRL) was added. Cells were incubated at 37°C with shaking (150 r.p.m.) for 1.5 h. The transformation mix ($50 \mu\text{l}$) was then serially diluted into K_0 buffer and three $5 \mu\text{l}$ aliquots were spotted from each dilution onto LK plates (Rowland *et al.*, 1984) supplemented with the appropriate antibiotic (as defined in the text). After an overnight incubation, the number of colonies present in each of the three spots was counted and then averaged. The transformation efficiency was determined as the number of transformants per μg of plasmid DNA.

Genomic DNA preparations and agarose gels

Exponential phase cells were exposed to either methylglyoxal (as defined) or cAMP and then a volume of culture, equivalent to 180 ml of an OD_{650} 0.05 ($\approx 7 \times 10^9$ cells), was filtered (Whatman, $0.45 \mu\text{m}$). The cells were resuspended in 50 ml K_0 , centrifuged ($4500 \times g$, 15 min) and then the cell pellets stored at -80°C until required. The DNA was isolated using a standard method (Qiagen), except that the incubation at 37°C was extended to 2.5 h. Purification of the genomic DNA was achieved using 100/G genomic tips (Qiagen). The DNA pellets were resuspended in $200 \mu\text{l}$ MilliQ water by an overnight incubation at 37°C , and then $4 \mu\text{l}$ was analysed on a 1% Tris acetate–EDTA agarose gel.

Pulsed-field gels

The pulsed-field gels were performed using a modification of a previously described method (Mattimore and Battista, 1996).

Exponential phase cells were treated with methylglyoxal (as defined) and then a volume, equivalent to 145 ml OD₆₅₀ 0.05 ($\approx 5.8 \times 10^9$ cells), was filtered (Whatman, 0.45 μm). The cells were resuspended in 50 ml K₀ buffer and then pelleted by centrifugation (4500 $\times g$, 15 min). After removal of the supernatant, the cells were resuspended in 1 ml K₀ buffer and then 126 μl of culture was centrifuged (microfuge, 14 000 $\times g$, 1 min). The cells were then resuspended in 0.5 ml 0.5 M EDTA (pH 8.0), mixed by vortexing, incubated at 65°C for 30 min and then recentrifuged (as above). The supernatant was discarded, 150 μl of 50 mM EDTA (pH 8.0) was added, the tubes vortexed and then an equal volume of 1.6% agarose (Sigma Type 1-A, low EEO) was added. After vortexing, this mix was pipetted into sterile plastic tubing (2 mm diameter) and left to set for 10 min. The agarose plug was then expelled, by exerting pressure using a 1 ml syringe, into 0.5 ml of 2 mg ml⁻¹ lysozyme in 50 mM EDTA and incubated at 42°C overnight. The solution was then replaced by 0.5 ml of 2 mg ml⁻¹ proteinase K in NDS (10 mM Tris-Cl, pH 8.0, 1% laurylsacrosine), incubated overnight at 42°C and then the agarose plugs were stored in 0.5 ml of 50 mM EDTA (pH 8.0) at 4°C until required.

Before restriction digestion of the agarose plugs, excess proteinase K was removed by treatment with 0.5 mg ml⁻¹ Pefabloc SC in TE (pH 7.0) according to the standard procedure (Boehringer Mannheim). The cells within the agarose plugs were then digested overnight at 37°C in 0.3 ml reaction volumes containing 3 μl 100 \times BSA, 30 μl 10 \times buffer and 2 μl *NotI* (10 000 U ml⁻¹). After digestion, the plugs were used immediately. The agarose plugs were cut to 5 mm, loaded into the wells of a 1% agarose gel (Sigma, Type 1-B, low EEO) in 0.5 \times Tris borate-EDTA and then the wells were sealed with 0.8% agarose (Sigma, Type 1-A, low EEO). The gel was run for 22 h at 6 V cm⁻¹, using initial and final ramp times of 10 and 60 s respectively (Chef-DR III, Bio-Rad). A lambda DNA ladder (starting at 48.5 kb and increasing by the same amount up to 1000 kb), embedded in 0.7% low-melt agarose, was also run on every gel (Bio-Rad).

DAPI staining

The samples were prepared as for the pulsed-field gels, except after the first centrifugation step the cells were resuspended in 100 μl of 1% (v/v) toluene, vortexed and stored at 4°C. When required, the cells were diluted by the addition of 900 μl of dilution buffer (10 mM NaCl, 6.6 mM Na₂SO₄, 5 mM HEPES, pH 7.0), and 33 μl of this was used for the assay. The DAPI assay was conducted according to a previously described procedure (Johnson, 1994), except that the cells were incubated with DAPI overnight at 4°C. The fluorescence intensity was measured with excitation and emission at 350 and 450 nm respectively.

Radiolabelling of genomic DNA with methyl-[³H]-thymidine

The genomic DNA was labelled with methyl-[³H]-thymidine using a modification of a previously described method (Davies *et al.*, 1973). An overnight culture in K_{0.2} medium was diluted 20-fold into fresh medium supplemented with 5 $\mu\text{Ci ml}^{-1}$ (0.1 μM) methyl-[³H]-thymidine and grown to an OD₆₅₀ of 0.4.

The culture was then filtered (Whatman, 0.45 μm), washed with K₀ buffer and then resuspended to the same OD₆₅₀ in K₀ buffer supplemented with 80 μM cold thymidine for 15 min. An aliquot (20 ml) was centrifuged (4500 $\times g$, 15 min), washed once with K₀ buffer and then resuspended in K_{0.2} medium to an OD₆₅₀ 0.05. Methylglyoxal was added to the amount defined in the text. To determine the radioactivity present in the low molecular weight fraction of the cells at a specified time, a volume equivalent to 180 ml of OD₆₅₀ 0.045 ($\approx 6.5 \times 10^9$ cells) was filtered, washed with K₀ buffer, resuspended in 30 ml of K₀ buffer and then centrifuged (as above). The pellet was resuspended in 0.5 ml of water, the cells lysed by boiling for 10 min and then the supernatant passed through a Nanosep (Flowgen) 3K microconcentrator (microfuge, 14 000 $\times g$, 20 min). A 3K microconcentrator will only allow material with a molecular weight <3000 to pass through into the filtrate. The filtrate (200 μl) was combined with 3 ml of scintillation fluid (Ultima Gold) and the level of ³H label quantified using scintillation counting. The radioactivity in the medium after methylglyoxal addition was determined by filtering 1 ml of culture (Whatman, 0.2 μm) at the defined time and the ³H-label level in the filtrate determined as above. To quantify the amount of radioactivity present in the low molecular weight material, the medium filtrate was also passed down a Nanosep 3K microconcentrator before counting.

Reproducibility

Although similar trends were always observed on different days, the actual viability data and amount of genomic DNA extracted varied. For this reason, all experiments shown here are a representative data set and have been replicated at least twice. Where shown, the error bars represent the standard deviation from the mean for data obtained from one experiment.

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