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& Cell Biologyjournal homepage: www.elsevier.com/locate/biocielGlyceraldehyde-3-phosphate dehydrogenase is required for efficient repair of cytotoxic DNA lesions in *Escherichia coli*Elaine Ferreira¹, Rosa Giménez¹, María Alexandra Cañas, Laura Aguilera, Juan Aguilar, Josefa Badia, Laura Baldomà*

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ABSTRACT

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional protein with diverse biological functions in human cells. In bacteria, moonlighting GAPDH functions have only been described for the secreted protein in pathogens or probiotics. At the intracellular level, we previously reported the interaction of *Escherichia coli* GAPDH with phosphoglycolate phosphatase, a protein involved in the metabolism of the DNA repair product 2-phosphoglycolate, thus suggesting a putative role of GAPDH in DNA repair processes. Here, we provide evidence that GAPDH is required for the efficient repair of DNA lesions in *E. coli*. We show that GAPDH-deficient cells are more sensitive to bleomycin or methyl methanesulfonate. In cells challenged with these genotoxic agents, GAPDH deficiency results in reduced cell viability and filamentous growth. In addition, the *gapA* knockout mutant accumulates a higher number of spontaneous abasic sites and displays higher spontaneous mutation frequencies than the parental strain. Pull-down experiments in different genetic backgrounds show interaction between GAPDH and enzymes of the base excision repair pathway, namely the AP-endonuclease Endo IV and uracil DNA glycosylase. This finding suggests that GAPDH is a component of a protein complex dedicated to the maintenance of genomic DNA integrity. Our results also show interaction of GAPDH with the single-stranded DNA binding protein. This interaction may recruit GAPDH to the repair sites and implicates GAPDH in DNA repair pathways activated by profuse DNA damage, such as homologous recombination or the SOS response.

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1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a key glycolytic enzyme, is a moonlighting protein with additional functions that are unrelated to its original metabolic role. The multifunctionality of GAPDH is extensively documented in human cells, where it is involved in numerous processes (Sirover, 2011). The various moonlighting functions of GAPDH depend on

Abbreviations: AP sites, abasic sites (apurinic/aprimidinic); BER, base excision repair; BM, bleomycin; CAA, casein acid hydrolysate; CFUs, colony-forming units; Endo, endonuclease; Exo, exonuclease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gph, phosphoglycolate phosphatase; GST, glutathione-S-transferase; HR, homologous recombination; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria-Bertani broth; MMS, methyl methanesulfonate; NIR, nucleotide incision repair; NTA, nitrilotriacetic acid; PTasRNA, paired termini antisense RNA; Rifampicin, Rif; SSB, single-stranded DNA binding protein; SM, minimal medium; UDG, uracil DNA glycosylase.

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specific post-translational modifications, oligomeric state and/or subcellular localization, which determine the binding partners (Copley, 2012; Sirover, 2005, 2011). Each function requires binding into specific protein complexes.

In bacteria, GAPDH moonlighting functions are mainly associated with its extracellular location. This protein is secreted and exposed on the bacterial surface, enabling pathogens to colonize and manipulate host cells (Pancholi and Chhatwal, 2003). In enteropathogenic and enterohemorrhagic *Escherichia coli*, secreted GAPDH interacts with human plasminogen and fibrinogen and remains associated with Caco-2 cells upon infection (Egea et al., 2007). In addition, the secreted protein can act as a target of oxidation and protect bacteria against the host oxidative response (Aguilera et al., 2009). GAPDH has also been identified in the secretome of probiotic strains (Aguilera et al., 2012; Sánchez et al., 2009a) and shown to interact with mucin, thus contributing to intestinal colonization (Kinoshita et al., 2008; Sánchez et al., 2009b).

Studies dealing with new GAPDH intracellular functions are scarce in bacteria. Following a proteomic approach aimed at identifying proteins that interact with GAPDH in *E. coli*, we identified

phosphoglycolate phosphatase (Gph) (Ferreira et al., 2013). This enzyme is involved in the metabolism of 2-phosphoglycolate formed in the DNA repair of 3'-phosphoglycolate ends generated by bleomycin (BM) (Pellicer et al., 2003). Interaction between Gph and GAPDH increases in BM-treated cells, thus implicating GAPDH in processes linked to DNA repair (Ferreira et al., 2013).

Involvement of GAPDH in the repair of BM-generated DNA lesions has been reported in human cells. GAPDH interacts with and modulates the activity of endonuclease APE1, an enzyme involved in the repair of abasic sites (AP sites) (Azam et al., 2008). AP sites are generated when cells are exposed to genotoxic agents such as BM or alkylating agents like methyl methanesulfonate (MMS).

BM is a radiomimetic drug that promotes site-specific free radical attack on deoxyribose moieties, generating AP sites as well as single- and double-strand breaks with mainly 3'-phosphoglycolate ends (Povirk, 1996). The AP sites are repaired through the base-excision repair (BER) pathway by the sequential action of AP-endonucleases (Endo IV and Exo III), DNA polymerase I and DNA ligase (Morita et al., 2010). The 3'-phosphoglycolate ends are removed as free 2-phosphoglycolate by Endo IV and Exo III before the strand breaks are repaired by the homologous recombination pathway (Fig. S1). MMS mainly produces methylations in several positions of purine bases (Sedgwick, 2004; Sedgwick et al., 2007). The major base modifications are N⁷-methylguanine or N³-methyladenine. These lesions are an important source of AP sites, which are mainly generated during their repair by the DNA glycosylases Tag or/and AlkA. O⁶-methylated guanines, also produced as minor products, are directly repaired by the alkyltransferases Ogt and Ada (Fig. S1).

The BER pathway is also involved in the processing of AP sites generated during the repair of spontaneous DNA lesions. One source of such AP sites is the action of uracil DNA glycosylases (UDG). These enzymes remove uracil present in DNA either as a product of cytosine deamination or mistaken incorporation of dUMP during replication (Morita et al., 2010).

Here, we report that *E. coli* GAPDH is required for the efficient repair of DNA lesions induced by BM or MMS, as well as of spontaneous AP sites. Deficiency in GAPDH leads to increased sensitivity to these agents and changes in cell morphology, which are consistent with the induction of the SOS response. Using pull-down assays with recombinant proteins expressed in different mutant backgrounds, we show interaction between GAPDH and proteins of the BER pathway, specifically Endo IV and UDG, as well as between GAPDH and SSB.

2. Materials and methods

2.1. Bacterial strains, growth conditions and preparation of cell extracts

The *E. coli* strains and plasmids used are listed in Table 1. Bacterial cells were routinely grown at 37°C in Luria-Bertani broth (LB), except the *gapA* mutant strains, which were grown in minimal medium (SM) with malate and glycerol as carbon sources (Ganter and Plückthun, 1990). In drug challenge experiments, cells were grown in SM with 0.5% casein acid hydrolysate (CAA) as carbon source (Pellicer et al., 2003). For *gapA* mutants, this medium was supplemented with 20 mM glycerol. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). When required, tetracycline (12.5 µg/ml), chloramphenicol (30 µg/ml), ampicillin (100 µg/ml) or rifampicin (Rif) (50 µg/ml) was added to the medium. Genetic crosses were performed by P1 transduction (Miller, 1992).

To construct strain MC4100 $\Delta gapA$, the $\Delta gapA::tet$ mutation present in strain W3CG was transduced into strain MC4100. Since

GAPDH-deficient mutants do not grow in LB, strain W3CG was first transformed with plasmid pBAD-*gapA* (Ferreira et al., 2013). Expression of GAPDH-V5 from this recombinant plasmid restored GAPDH deficiency and allowed strain W3CG to grow in this medium. The P1 lysate obtained from this transformed strain was used to infect strain MC4100, and transductants were selected on SM-malate-glycerol plates containing tetracycline. The correct insertion of $\Delta gapA::tet$ in the transductants was assessed by negative growth on SM-glucose plates. One of these transductants, strain MC4100 $\Delta gapA$, was selected and GAPDH deficiency was demonstrated by enzymatic activity and western blot analysis.

Cell extracts were obtained by sonic disruption of bacterial cells as described previously (Aguilera et al., 2012).

2.2. Recombinant DNA techniques

Bacterial genomic DNA was obtained using the Wizard Genomic DNA purification kit (Promega), and plasmid DNA was prepared using the Wizard Plus SV Midipreps DNA purification system (Promega). DNA manipulations were performed essentially as described elsewhere (Sambrook and Russell, 2001). DNA fragments were amplified by PCR using *E. coli* chromosomal DNA as a template. Primers used in this work are listed in Table S1.

To obtain recombinant GST-Ogt, the *ogt* gene was amplified by PCR and cloned into *Bam*HI/*Eco*RI restriction sites of vector pGEX-3x (Amersham) yielding plasmid pGEX-Ogt. Recombinant His₆-Exo III was produced from plasmid pQE30-XthA, which was constructed by cloning the PCR-amplified *xthA* gene into *Bam*HI/*Hind*III restriction sites of vector pQE30 (Qiagen).

2.3. Cloning of *gapA* antisense RNA (asRNA) sequences into IPTG-inducible vectors for conditional gene silencing

To obtain *gapA* asRNA, we used a collection of IPTG-inducible expression vectors that allow conditional expression of the asRNA with paired termini (PTasRNA), thus conferring great RNA stability and high silencing efficacy (Nakashima et al., 2006; Nakashima and Tamura, 2009). Two sets of oligonucleotides were used to amplify two different *gapA* asRNA sequences (Table S1). Both asRNAs were complementary to the genomic region that encompasses the *gapA* ribosome binding site and the start codon. The amplified PCR products were cloned into the *Nco*I/*Xho*I restriction sites of the PTasRNA vectors pHN1009, pHN678 or pHN1242, which differ in their copy number (Table 1). Strain XL1-Blue was used for cloning and plasmid preparation. Strain MC4100 was used as a host for expressing *gapA* PTasRNA in silencing experiments. To this end, cells of strain MC4100 bearing the *gapA* PTasRNA constructs were grown overnight in the absence of IPTG. These cultures were diluted 1:200 with fresh medium containing 1 mM IPTG and cultured to mid- to late-logarithmic phase depending on the experiment. Knockdown GAPDH expression under these conditions was assessed by measurement of enzyme activity and western blot analysis in cell extracts.

2.4. Expression and purification of recombinant proteins

Recombinant wild-type GAPDH and the derived C149A and C153A mutants were expressed and purified using the glutathione-S-transferase (GST) gene fusion system with recognition sites for factor Xa cleavage, as described elsewhere (Egea et al., 2007; Aguilera et al., 2009). This expression system was also used to obtain recombinant Ogt. For this protein, induction and purification conditions were the same as those described for GST-GAPDH.

Proteins Endo IV (Nfo), single-stranded DNA binding protein (SSB), 6-methylguanine-DNA methyltransferase (Ada), 3-methyladenine DNA glycosylases I (Tag) and II (AlkA) and uracil

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
<i>E. coli</i> strains		
XL1Blue	<i>recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1</i> (F' <i>proAB lacI^q lacZ ΔM15 Tn10</i>)	Stratagene
BL21(DE3)	F ⁻ <i>ompT hsd(r_B⁻ m_B⁻) gal</i>	Amersham
M15[pREP4]	F ⁻ <i>NaI^s Str^s rif^s lac⁻ mtl⁻ recA⁻</i> [pREP4 Kan ^r]	Qiagen
AG1 (ME5053)	<i>recA1 endA1 gyrA96 thi-1 hsdR17(r_K⁻ m_K⁻) supE44 realA1</i> (host cell for pCA24N)	NBRP; Kitagawa et al. (2005)
W3CG	F ⁻ λ <i>thyA36 deoC2 IN(rrnD-rrnE)</i> (W3110) Δ <i>gapA::tet supE</i>	Ganter and Plüctthun (1990)
MC4100	F ⁻ <i>araD Δ(argF-lac) rpsL(Str^r) relA fihD deoC ptsF rbs</i>	Casadaban, 1976
MC4100 Δ <i>gapA</i>	MC4100 Δ <i>gapA::tet</i>	This study
MG1655 <i>ung::kan</i>	MG1655 <i>ung::kan</i>	Bharti and Varshney (2010)
MG1655 Δ <i>nfo::cm</i>	MG1655 Δ <i>nfo::cm</i>	Bjedov et al. (2007)
Plasmids		
pGEX-3x	Vector for expression of N-terminal GST-tag proteins, Ap ^r	Amersham
pGEX-GapA	pGEX-3x expressing GST-GAPDH, Ap ^r	Egea et al. (2007)
pGEX-GapA.C149A	pGEX-3x expressing GST-GAPDH C149A mutant, Ap ^r	Aguilera et al. (2009)
pGEX-GapA.C153A	pGEX-3x expressing GST-GAPDH C153A mutant, Ap ^r	Aguilera et al. (2009)
pGEX-Ogt	Gene <i>ogt</i> cloned in pGEX-3x for expression of GST-Ogt	This study
pBAD-GapA	Gene <i>gapA</i> cloned in pBAD-TOPO for expression of GAPDH-V5	Ferreira et al. (2013)
pQE30	Vector for expression of N-terminal His ₆ -tag proteins, Ap ^r	Qiagen
pQE30-XthA	pQE30 expressing His ₆ -ExoIII, Ap ^r	This study
JW2146	ASKA clone for expression of His ₆ -EndoIV (<i>nfo</i> gene cloned in pCA24 N), Cm ^r	NBRP; Kitagawa et al. (2005)
JW4020	ASKA clone for expression of His ₆ -SSB (<i>Ssb</i> gene cloned in pCA24 N), Cm ^r	NBRP; Kitagawa et al. (2005)
JW3518	ASKA clone for expression of His ₆ -Tag (<i>tag</i> gene cloned in pCA24 N), Cm ^r	NBRP; Kitagawa et al. (2005)
JW2201	ASKA clone for expression of His ₆ -Ada (<i>ada</i> gene cloned in pCA24 N), Cm ^r	NBRP; Kitagawa et al. (2005)
JW2053	ASKA clone for expression of His ₆ -AlkA (<i>alkA</i> gene cloned in pCA24 N), Cm ^r	NBRP; Kitagawa et al. (2005)
pHN678	Vector for expression of PT-asRNA, medium copy number (pACYC ori), Cm ^r	Nakashima and Tamura (2009)
pHN1009	Vector for expression of PT-asRNA, high copy number (pBR322 ori), Ap ^r	Nakashima and Tamura (2009)
pHN1242	Vector for expression of PT-asRNA, low copy number (pSC101 ori), Kan ^r	Nakashima and Tamura (2009)
pHN1009-1	pHN1009 expressing <i>gapA</i> -asRNA (-76/+86), Ap ^r	This study
pHN1009-2	pHN1009 expressing <i>gapA</i> -asRNA (-97/+70), Ap ^r	This study

DNA glycosylase (UDG) were expressed as His₆-tagged proteins from the ASKA clones JW2146, JW4020, JW2201, JW3518, JW2053 and JW2564, respectively (NBRP, *E. coli* Strain National BioResource Project) (Kitagawa et al., 2005). The host strain for ASKA clones was AG1 (ME5053) (Table 1). The recombinant protein His₆-ExoIII was expressed from plasmid pQE30-XthA in strain M15 [pREP4] (Table 1). Expression of the His-tagged proteins was induced by addition of 0.1 mM IPTG to exponential grown cultures, followed by overnight incubation at 20 °C. Proteins were purified under native conditions with Ni²⁺-nitrilotriacetic acid (NTA) resin (Qiagen), as described elsewhere (Kitagawa et al., 2005).

2.5. Pull-down assays and immunoblotting analysis

Pull-down assays based on His₆-tagged recombinant proteins were performed essentially as described previously (Aguilera et al., 2012). GST pull-down assays were performed to analyze interaction of GST-GAPDH (either the wild-type protein or the derived C149A or C153A mutants) with His₆-Endo IV. In these experiments, expression and binding of GST-GAPDH to a glutathione-Sepharose 4B column was carried out as previously described (Egea et al., 2007; Aguilera et al., 2009). At this point, column-bound GST-GAPDH, equilibrated in PBS buffer, was incubated for 30 min at 4 °C with 0.5 ml of a cell extract (10 mg/ml) obtained in PBS buffer from AG1 cells expressing His₆-EndoIV. After extensive washing, recombinant GAPDH and interacting proteins were eluted with glutathione elution buffer and analyzed by SDS-PAGE (Laemmli, 1970) followed by immunoblotting analysis with anti-His antibodies. Pull-down experiments with the GST-tag were performed in parallel as a negative control.

Western blot analysis was performed as described previously (Egea et al., 2007; Aguilera et al., 2012).

2.6. Drug exposure and cell survival

MC4100 cells bearing plasmid pHN1009-1 for *gapA* silencing or the vector pHN1009 as a control (Table 1) were grown aerobically

to mid-exponential phase (OD₆₀₀ of 0.5) in SM-0.5% CAA supplemented with ampicillin and 1 mM IPTG. At this point, cultures were challenged with BM (10 μg/ml in the presence of 50 μM FeSO₄), MMS (10 mM) or H₂O₂ (2.5 mM). At different times, aliquots of the cultures were collected, washed twice with SM and processed for analysis. Survival was estimated by diluting cells in SM, followed by plating on LB-ampicillin plates to determine the number of colony forming units (CFUs). GAPDH activity was determined in cell extracts. In drug-challenge experiments using the knockout mutant strain MC4100 Δ *gapA*, the growth medium was SM-0.5% CAA supplemented with 20 mM glycerol and doses of BM and MMS were 5 μg/ml and 5 mM, respectively. Bleomycin sulfate (Almirall Prodesfarma, Barcelona, Spain) was freshly prepared with 50 mM phosphate buffer (pH 7.5) and FeSO₄ solutions were prepared immediately before use.

2.7. Microscopic observations

To examine the morphology of growing cells upon drug exposure, liquid cultures were processed as described above and treated with BM (10 μg/ml) or MMS (10 mM) for 10 and 20 min. Then, cultures were washed with PBS, suspended in fresh medium and incubated aerobically at 37 °C to re-start growth. After 3 h, aliquots were taken, spread onto glass slides, fixed, stained with methylene blue and examined under a light microscope (Leica DM1000) with a 100× objective lens. Non-treated bacteria were processed in parallel as a control.

2.8. Enzyme activities

GAPDH activity was measured following the reduction of NAD⁺ to NADH at 340 nm (Pancholi and Fischetti, 1992).

Endo IV activity was assayed coupled to uracil-DNA glycosylase (UDG) using a 40-mer duplex DNA substrate containing a U-G mismatch (U19.G) as described elsewhere (Shatilla and Ramotar, 2002; Bulgar et al., 2012). This

dsDNA oligonucleotide was prepared by mixing equimolar quantities of the two complementary oligonucleotides 5'(DIG)-GTAAAACGACGGCCAGTGUATT CGAGCTCGGTACCCGGGG-3' and 5'(DIG)-CCCCGGGTACCGAGCTCGAATGCACTGGCCGTCGTTTAC-3', both 5'-end labeled with digoxigenin (DIG) (Sigma Genosys). The mixture was heated to 80 °C for 30 min and then allowed to cool slowly overnight at room temperature. To create an AP site, the DIG-labeled duplex DNA (20 pmol) was incubated with commercial UDG (Sigma Aldrich) (2 units) at 37 °C for 1 h in 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1 mM DTT and 50 mM NaCl in a final volume of 20 µl. Aliquots of 4 µl containing the AP-site substrate were incubated with Endo IV (New England Biolabs) (2 units) at 37 °C for 30 min in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl₂, 0.1 mM DTT and 10 mM NaCl in a final volume of 20 µl. Reactions were stopped by transferring tubes on ice followed by the addition of an equal volume of formamide loading buffer (76% formamide, 0.3% bromophenol blue, 0.3% xylene cyanole and 10 mM EDTA) and incubation at 65 °C for 5 min. Reaction products (20-mer fragments) were resolved by electrophoresis on denaturing 10% polyacrylamide/7 M urea gel and blotted onto a nylon membrane using a Bio-Rad electroblotting system. Detection of DIG-labeled DNA fragments was carried out using the enzyme immunoassay DIG Luminescent Detection Kit (Roche). Reactions omitting UDG or Endo IV were performed in parallel as a control. When indicated, purified GAPDH (10 µg) was added instead of UDG. When used, the UDG inhibitor Ugi (New England Biolabs) was added to the reaction mixture containing UDG and buffer. After 10-min pre-incubation at 37 °C, reaction was started by the addition of the DIG-labeled duplex DNA substrate.

2.9. Quantification of AP sites in genomic DNA

Bacterial cultures were grown aerobically at 37 °C to an OD₆₀₀ of 0.5. Cells were collected by centrifugation and subjected to DNA extraction using the DNAzol[®] Reagent (Invitrogen). DNA was quantified with a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies). Quantification of AP sites was performed by using the Oxiselect[™] Oxidative DNA Damage Quantification Kit (Cell Biolabs) according to the manufacturer's protocol.

The presence of AP sites in DNA was also evaluated by means of their susceptibility to alkaline pH as described elsewhere (Bharti and Varshney, 2010). Genomic DNA (1 µg) was treated with 0.1 N NaOH, heated at 90 °C for 10 min and analyzed by electrophoresis on 1% agarose gel containing ethidium bromide.

2.10. Determination of mutation frequencies

Five independent cultures of each strain were grown in SM-0.5% CAA-20 mM glycerol to an OD₆₀₀ of 0.4–0.5. At this point, cells were harvested and resuspended in 100 µl of the same culture medium, plated on SM-CAA-glycerol agar containing Rif (50 µg/ml) and incubated overnight at 37 °C in dark. Rif-resistant colonies were scored. For each sample, the total number of viable cells was estimated by serial dilution followed by colony counting on SM-CAA-glycerol plates.

2.11. Quantification of cellular ATP levels

Bacterial cells were grown aerobically to an OD₆₀₀ of 0.5 and cellular ATP concentrations were measured using the ATP bioluminescence Assay Kit HS II (Roche) according to manufacturer's instructions.

3. Results

3.1. Sensitivity of GAPDH-deficient cells to DNA-damaging agents

To test whether *E. coli* GAPDH performs a moonlighting function in DNA repair, we examined the sensitivity of the *gapA* null mutant (MC4100 $\Delta gapA$) to agents that damage DNA, specifically BM and MMS. The parental strain MC4100 was processed in parallel as a control. Drug treatment was performed in cells grown aerobically to mid-exponential phase in SM-0.5% CAA supplemented with 20 mM glycerol. In this medium, no differences in cell growth were observed between the $\Delta gapA$ mutant and its parental strain (Fig. S2A). At different times of incubation with these compounds, cell survival was evaluated. GAPDH-deficient cells were more sensitive than the wild-type cells to both BM and MMS (Fig. 1).

Since GAPDH has a central role in bacterial metabolism, we approached a parallel analysis using *E. coli* cells with reduced GAPDH levels to avoid possible side effects of complete GAPDH deficiency. To knockdown GAPDH levels, we prepared several constructs (high-, medium- and low-copy number) that allow IPTG-inducible expression of two *gapA* asRNA, (-76/+86) or (-97/+70). The effect of *gapA* asRNA expression on cell growth and GAPDH protein levels was analyzed in LB-IPTG cultures of transformed MC4100 cells. From this screening, we selected the construct pHN1009-1, which expressed the *gapA* asRNA (-76/+86) from the high copy number vector pHN1009 (Fig. S3). Before approaching drug challenge experiments, we confirmed that expression of *gapA* asRNA from pHN1009-1 did not impair growth of strain MC4100 in SM-0.5% CAA. Under these conditions, the level of GAPDH protein, monitored by enzyme activity and immunoblotting, was reduced by nearly 90%, as compared with cells bearing the vector pHN1009 (Fig. S2). The *gapA*-silenced cells (MC4100 bearing pHN1009-1) were challenged with BM or MMS, and also with H₂O₂. Cell viability was estimated after different times of incubation and compared with that of cells bearing vector pHN1009. Results showed that *gapA*-silenced cells were more sensitive than wild-type cells to BM and MMS, especially at incubation times up to 60 min (Fig. 2A). In contrast, no differences in cell survival were observed between wild-type or *gapA*-silenced cells treated with H₂O₂ at any incubation time (Fig. 2B). H₂O₂ inhibits GAPDH activity by oxidation of catalytic C149, which forms a disulphide bound with C153 (Leichert et al., 2008). Consistently, GAPDH levels of MC4100 (pHN1009) cells treated with H₂O₂ were significantly reduced, being around 15% of that of the non-treated cells (Fig. 2C). However, exposure to BM or MMS did not modify GAPDH activity. Wild-type as well as silenced cells displayed similar GAPDH activity levels to the corresponding non-treated controls (Fig. 2C), which indicated that neither genotoxic agent oxidizes the catalytic C149 residue. This observation is compatible with the action mechanism of these compounds (Sedgwick et al., 2007; Povirk, 1996). Overall, these results suggested that the role of GAPDH in DNA repair may depend on the reduced state of C149.

3.2. Filamentous growth of GAPDH-deficient cells after BM or MMS treatment

The SOS response is a bacterial defense system activated under conditions of extensive DNA damage. Induction of the SOS response results in filamentous growth due to the expression of gene *sulA*, which encodes an inhibitor protein of cell division. Under these conditions, delay in cell division allows for DNA repair after treatment with DNA-damaging agents (Janion et al., 2003; Janion, 2008). Since *gapA*-knockout and *gapA*-silenced cells were more sensitive to BM and MMS than the wild-type strains, we analyzed cell morphology at 3-h growth in fresh medium after 10- or 20-min treatment with these DNA-damaging agents. After

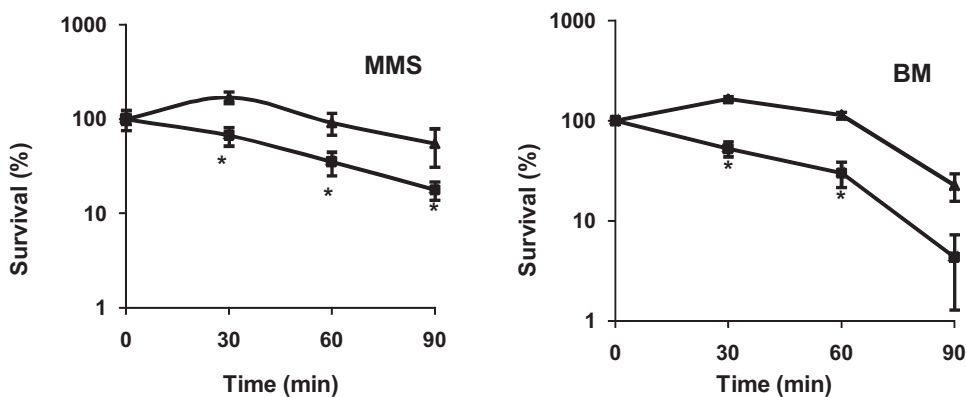


Fig. 1. Effect of GAPDH deficiency on cell survival after treatment with DNA-damaging agents. Cells of MC4100 $\Delta gapA$ (squares) and its parental strain MC4100 (triangles) grown in SM-0.5% CAA + 20 mM glycerol to an $OD_{600\text{ nm}}$ of 0.5 were challenged with 5 mM MMS or 5 $\mu\text{g/ml}$ BM-50 μM de FeSO_4 . At the indicated times, aliquots were collected and monitored for cell survival. Values, given as the percentage of viable cells (CFUs on SM-CAA-glycerol plates) with respect to non-treated cells, are the mean \pm standard deviation (SD) of three replicates. * $P < 0.05$ compared to wild-type cells by ANOVA followed by Tukey's test.

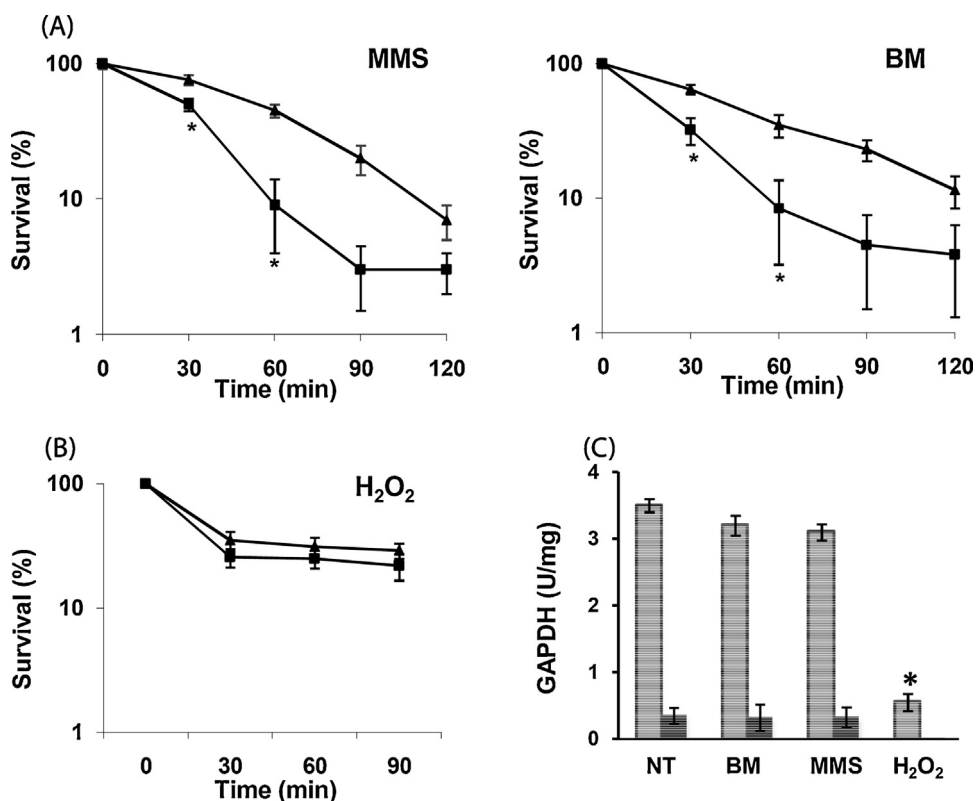


Fig. 2. Effect of *gapA*-silencing on cell survival after treatment with DNA-damaging agents. Control (pHN1009 in triangles) and *gapA*-silenced (pHN1009-1 in squares) MC4100 cells were challenged with: (A) 10 $\mu\text{g/ml}$ BM-50 μM de FeSO_4 or 10 mM MMS and (B) 2.5 mM H_2O_2 . At the indicated times, aliquots were collected and monitored for cell survival. Values are given as the percentage of viable cells (CFUs on SM-CAA ampicillin plates) with respect to non-treated cells. (C) Values of GAPDH activity measured in cell extracts obtained from silenced cells bearing pHN1009-1 (black bars) or from cells bearing pHN1009 vector (gray bars) after 2-h treatment with the indicated genotoxic agents. The values are the mean \pm standard deviation (SD) of three replicates. * $P < 0.05$ compared to wild-type cells by ANOVA followed by Tukey's test.

3-h recovery, the wild-type cells displayed normal growth while GAPDH-deficient cells formed filaments when treated either with MMS or BM (Fig. 3). These results are compatible with involvement of GAPDH in the repair of MMS- and BM-generated DNA damage in *E. coli*.

AP sites are commonly formed in response to both genotoxic agents. To analyze the level of unrepaired DNA in wild-type and $\Delta gapA$ cells after 3-h recovery from 10 mM MMS-treatment, we performed a genomic degradation assay (Bharti and Varshney, 2010). This protocol was based on the susceptibility of AP sites to alkaline pH. Samples obtained before and after 20-min treatment were processed in parallel for comparison. Genomic DNA was

treated with NaOH and heated to 90 °C. The migration rate of the denaturalized DNA species was evaluated by gel electrophoresis (Fig. 4). Assays performed with DNA samples obtained after 20 min treatment resulted in the formation of fast-migrating degradation products in both cells (Fig. 4, lanes 2 and 5). After 3-h recovery in fresh medium, most of the denaturalized DNA from strain MC4100 corresponded to high molecular weight species (Fig. 4, lane 3), whereas in the case of the $\Delta gapA$ mutant, these species were less abundant. In addition, defined degradation fragments were more apparent (Fig. 4, lane 6). These results were consistent with the presence of more unrepaired DNA lesions in the *gapA* null mutant than in the wild-type strain.

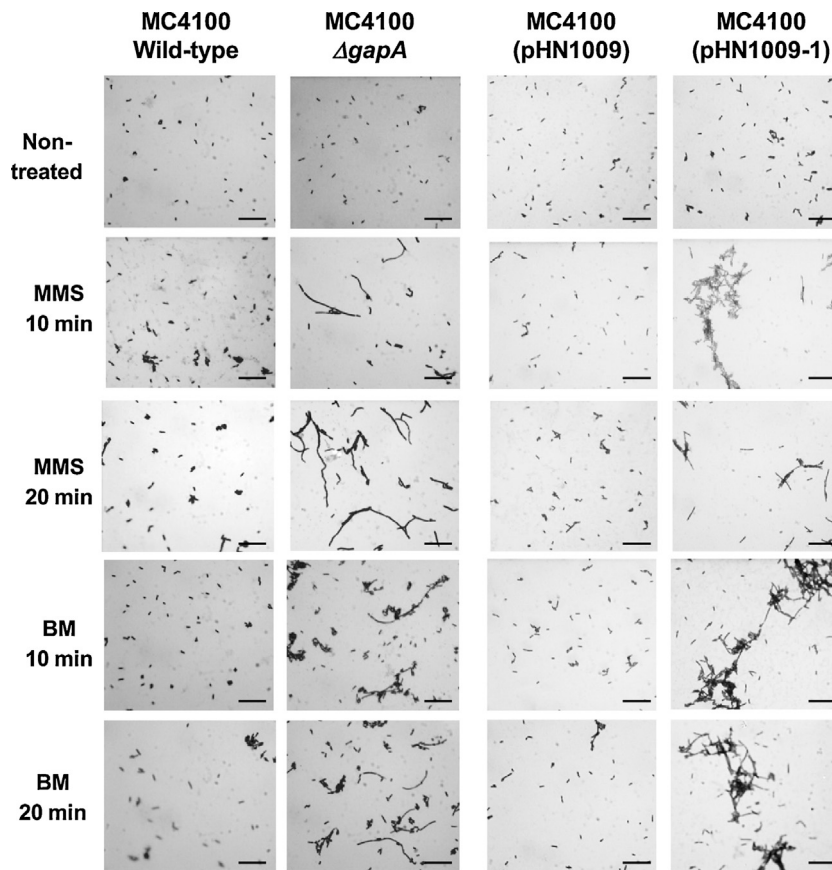


Fig. 3. Filamentous growth of GAPDH-deficient cells after treatment with MMS or BM. Bacteria were treated with MMS (10 mM) or BM (10 μ g/ml) for 10 or 20 min, washed and further incubated in the growth medium for 3 h. Raw 1 presents bacteria before exposure to these DNA-damaging agents. Bar = 10 μ m.

3.3. Accumulation of spontaneous AP sites in *gapA* knockout mutants

The alkali degradation assay performed with genomic DNA isolated from cells before MMS addition (Fig. 4, lanes 1 and 4) showed

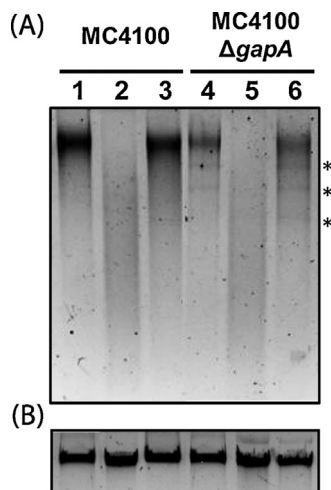


Fig. 4. Genome degradation assays after alkaline treatment. Exponential cultures of the indicated cells were challenged with 10 mM MMS for 20 min and let to recover in fresh medium as described in Fig. 3. DNA was isolated from cells before MMS treatment (lanes 1 and 4), after MMS treatment (lanes 2 and 5) and after 3-h recovery in fresh medium (lanes 3 and 6). (A) Genomic DNA (1 μ g) was heated in the presence of NaOH and analyzed by electrophoresis on agarose gel containing ethidium bromide. Prominent DNA degradation fragments are indicated by asterisks. (B) For each condition, non-treated genomic DNA (1 μ g) was resolved by electrophoresis as a loading control.

Table 2

Effect of GAPDH deficiency on the repair of spontaneous DNA lesions.

Strain	AP sites/ 100,000 bp	Mutation frequency ($\times 10^{-9}$)	Cellular ATP levels (pmol/ 10^6 cells)
MC4100	8.5 \pm 0.4	11.4 \pm 3.1	4.1 \pm 0.3
MC4100 $\Delta gapA$	12.8 \pm 0.8	54.2 \pm 5.2	3.9 \pm 0.2

Cells were grown in SM-0.5% CAA supplemented with 20 mM glycerol to an OD_{600 nm} of 0.5 and processed as described in Section 2 for each assay.

a minor amount of high-molecular mass products in DNA samples from MC4100 $\Delta gapA$ than the wild-type strain. This observation suggested the presence of more spontaneous AP sites in the *gapA* mutant DNA. In fact, quantification of AP sites with a biotinylated aldehyde-reactive probe, followed by biotin detection coupled to streptavidin, showed a 1.5-fold increase in the number of spontaneous AP sites in the genomic DNA of the *gapA* null mutant when compared to the parental strain (Table 2).

Since AP sites are mutagenic, we determined mutation frequencies of wild-type MC4100 and MC4100 $\Delta gapA$ by scoring for Rif-resistant colonies on SM-0.5% CAA-20 mM glycerol plates. Consistently, the *gapA* mutant showed a 4.7-fold increase when compared with the wild-type strain (Table 2). Under the growth conditions used no differences in cellular ATP levels were observed between the two strains (Table 2). Thus, inefficient repair of spontaneous AP sites in the $\Delta gapA$ mutant may not be attributed to a reduced energy supply.

3.4. GAPDH interacts with Endo IV and SSB

Since moonlighting functions of GAPDH may depend on its ability to interact with other proteins and participate in protein

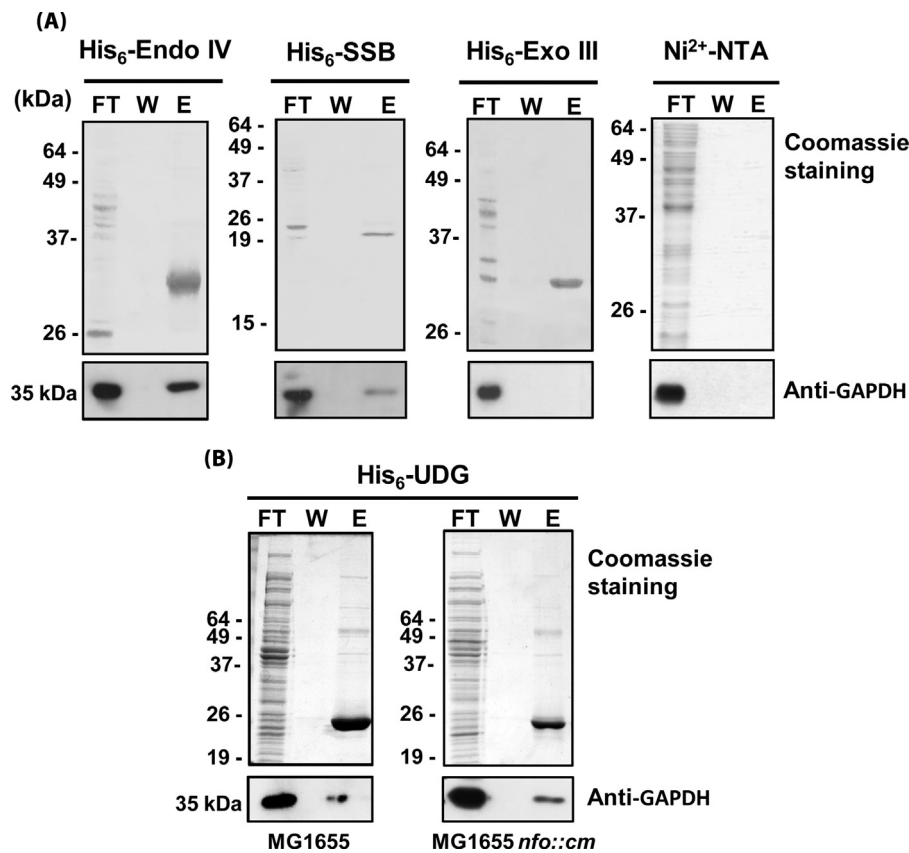


Fig. 5. Pull-down experiments to analyze interaction of GAPDH with proteins involved in DNA repair. (A) Cell extracts obtained from induced cultures of strain AG1 bearing the ASKA clone JW2146 (expressing His₆-Endo IV), ASKA clone JW4020 (expressing His₆-SSB) or pQE30-XthA (expressing His₆-Exo III) were applied to a Ni²⁺-NTA column. After extensive washing, elution of recombinant proteins was achieved with 300 mM imidazole (elution buffer). As a control, a cell extract of the host cell AG1 was applied to the Ni²⁺-NTA column and processed in parallel (right panel). Column fractions (FT: flow-through; W: wash; E: elution) were analyzed either by Coomassie blue staining (upper panels) or by western blot with anti-GAPDH antibodies. (B) Pull-down experiments to analyze interaction of GAPDH with UDG. The ASKA clone JW2564 (His₆-UDG) was expressed in an Endo IV-deficient background (MG1655 $\Delta nfo::cm$) as well as in the wild-type isogenic strain (MG1655) for comparison. Cell extracts were applied to a Ni²⁺-NTA column and processed as described above.

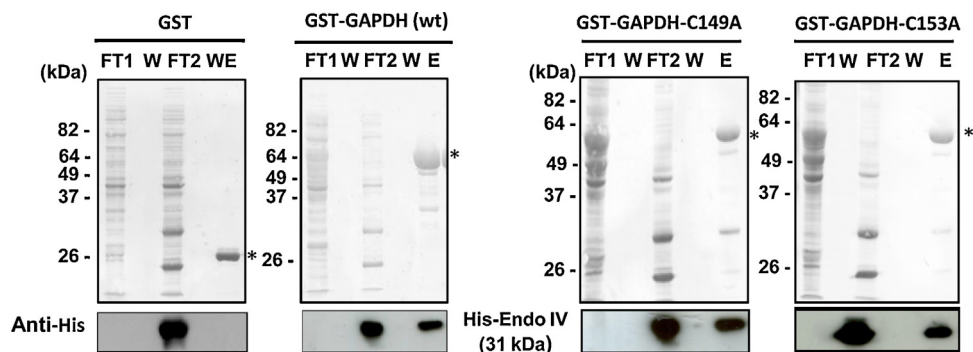


Fig. 6. Analysis of GAPDH-Endo IV interaction. Cells extracts of strain MC4100 $\Delta gapA$ expressing GST (as a control) or the indicated GST-GAPDH variants were applied to Glutathione-Sepharose 4B columns to immobilize these recombinant proteins. After washing, a cell extract obtained from induced cells bearing the ASKA clone JW2146 (expressing His₆-Endo IV) was passed over the columns. After extensive washing, the GST-tagged recombinant proteins were eluted with glutathione-elution buffer. Column fractions were analyzed either by Coomassie blue staining (upper panel) or by western blot with anti-His antibodies. FT1, first flow-through (before applying the cell extract expressing His₆-Endo IV); FT2, second flow-through (after applying the cell extract expressing His₆-Endo IV); W, final wash of each step; E, eluted fraction.

complexes dedicated to specific cellular functions, we next analyzed whether GAPDH could interact with proteins of the BER pathway like the AP-endonucleases Endo IV and Exo III, and the glycosylases Tag and AlkA. As filamentous growth of GAPDH-deficient cells revealed activation of the SOS response, we included SSB, an essential protein of this pathway that recruits and binds a variety of DNA repair proteins. In addition, we selected Ogt and Ada, enzymes involved in the direct repair of O⁶-methylguanine lesions, as Ogt had been identified as a putative binding partner of *E. coli*

GAPDH in large-scale protein interaction studies (Butland et al., 2005). All these proteins were expressed as His₆-tagged recombinant proteins and immobilized in a Ni²⁺-NTA resin, except Ogt, which was expressed as GST-Ogt fusion protein and bound to a glutathione-Sepharose 4B column. Pull-down experiments were then performed to examine retention of chromosome-encoded GAPDH. After extensive washing, GAPDH co-eluted with His₆-SSB and His₆-Endo IV as detected by western blot probed with anti-GAPDH specific antibody (Fig. 5A). No interaction between GAPDH

and Exo III (Fig. 5A), Tag, AlkA, Ada or Ogt was detected (not shown). To confirm interaction between GAPDH and Endo IV, pull-down experiments were performed using GST-GAPDH as bait. In this case, GST-GAPDH affinity resin was incubated with a cell extract obtained from *E. coli* cells overexpressing His₆-Endo IV. After extensive washing, retention of this endonuclease on the resin was assessed by western blot analysis of the eluted fraction probed with anti-His antibodies. Immunodetection of a 31 kDa protein in the eluted fraction from the GST-GAPDH affinity resin, but not from the control GST-bound resin, confirmed GAPDH-Endo IV interaction (Fig. 6). To test whether this interaction depends on the GAPDH residues C149 or C153, pull-down experiments were performed using as bait the cysteine variants GST-GAPDH C149A or GST-GAPDH C153A. To avoid interference with native GAPDH encoded in the chromosome, expression of these recombinant GAPDH variants was approached in strain MC4100 Δ gapA. Endo IV was also retained and co-eluted with these GAPDH mutants (Fig. 6).

3.5. GAPDH is a component of a multiprotein complex that repairs DNA lesions through the BER pathway

A moonlighting function described for human GAPDH is its activity as uracil-DNA glycosylase (UDG) (Meyer-Siegler et al., 1991). To analyze whether *E. coli* GAPDH displays UDG activity, a 5'-DIG-labeled 40-mer double-stranded DNA substrate containing a U-G mismatch (U19.G) was incubated with purified *E. coli* GAPDH (Egea et al., 2007) or with commercial UDG (Sigma Aldrich) as a positive control. Removal of uracil from this substrate was measured by coupling commercial *E. coli* Endo IV, which cleaves the AP-site-containing strand in two 20-mer products. Visualization of the 20-mer product in reactions incubated with both commercial UDG and Endo IV validated the coupled enzyme assay (Fig. 7A, lane 4). As expected, this product was not observed when UDG was omitted from the reaction mixture (Fig. 7A, lane 2). However, in the assays performed in the absence of Endo IV, a faint band corresponding to the 20-mer product was observed (Fig. 7A, lane 3), suggesting that the commercial UDG used here was contaminated with an AP-endonuclease, like Endo IV. Cleavage of the duplex oligonucleotide substrate was also produced when recombinant GAPDH was used instead of commercial UDG, either in the presence or absence of Endo IV. The same result was obtained with the two variants, GAPDH-C149A or GAPDH-C153A (Fig. 7A, lanes 5–10). These results suggested either that *E. coli* GAPDH displays UDG activity or that the UDG activity observed can be attributed to the chromosome-encoded UDG enzyme (*ung* gene product) that may co-purify with our recombinant GAPDH. To examine these two hypotheses, we measured the GAPDH-associated UDG activity in the presence of the *Bacillus subtilis* inhibitor Ugi, a protein that specifically inhibits UDG enzymes from various organisms (Putnam et al., 1999; Shatilla and Ramotar, 2002). Pre-incubation of commercial UDG (as a control) or purified GAPDH with this inhibitor prevented cleavage by Endo IV of the 40-mer substrate bearing U19.G mismatch, in both cases (Fig. 7B). These results suggested that the UDG activity observed with our GAPDH preparation may be due to the presence of chromosome-encoded UDG enzyme (Ung). To assess this hypothesis, we transformed the mutant strain MG1655 *ung::kan* with plasmid pGEX-GapA to express recombinant GST-GAPDH in a UDG-deficient background. Purification of GAPDH from this strain by affinity chromatography followed by factor Xa cleavage yielded an Ung-free GAPDH sample. When this sample was used as a source of UDG activity in the Endo IV-coupled assay, the 20-mer cleavage product was not observed (Fig. 7C, lanes 5 and 6), indicating that *E. coli* GAPDH does not display UDG activity. Thus, the UDG activity observed with GAPDH samples purified from *E. coli* strains with an intact *ung* gene was due to chromosome-encoded UDG. In addition, these results indicated interaction between GAPDH and the

UDG enzyme. To test whether interaction between the two proteins requires Endo IV, recombinant GST-GAPDH was expressed in an Endo IV deficient strain (MG1655 *nfo::cm*), purified by affinity chromatography, followed by factor Xa cleavage as described above, and used as a source of UDG activity in the Endo IV-coupled assay (Fig. 7C, lanes 7 and 8). As GAPDH does not display UDG activity, formation of the 20-mer product (Fig. 7, lane 8) may be attributed to the presence of chromosome-encoded UDG. Pull-down experiments were then performed to confirm interaction between GAPDH and UDG. Recombinant His₆-UDG was expressed in the *nfo* mutant as well as in its parental wild-type strain MG1655. Each batch of recombinant UDG protein was applied to a Ni²⁺-NTA resin and the presence of chromosome-encoded GAPDH in the elution fractions was analyzed by western blot (Fig. 5B). Co-elution of GAPDH with UDG in both cases confirmed the interaction between these proteins and indicated that this interaction does not depend on Endo IV.

The presence of the 20-mer product in the reactions performed with GAPDH in the absence of added Endo IV (Fig. 7A, lanes 5, 7 and 9) indicated that the GAPDH preparation also contained associated Endo IV. This finding was compatible with results presented above showing co-elution of both proteins in pull-down experiments in different affinity supports (Figs. 5A and 6). Immunodetection of GAPDH in the elution fractions of pull-down experiments performed using as bait His₆-Endo IV expressed in the mutant strain MG1655 *ugn::kan* (not shown) indicated that UDG is not required for GAPDH-Endo IV interaction.

4. Discussion

Moonlighting functions related with DNA repair have been reported for human GAPDH. This protein translocates to the nucleus when cells are challenged with oxidative stress conditions (Dastoor and Dreyer, 2001) and has been found associated with damaged DNA in cells treated with chemotherapeutic agents (Krynetski et al., 2003). In addition, human GAPDH interacts with the endonuclease APE1, an essential enzyme for the repair of AP sites. This interaction contributes to reactivation of oxidized forms of APE1 generated as a consequence of aerobic metabolism (Azam et al., 2008).

The study presented here in *E. coli* implicates GAPDH in the repair of DNA lesions in bacteria. Several lines of evidence support this conclusion: (i) increased sensitivity of GAPDH-deficient cells to BM or MMS, (ii) filamentous growth of GAPDH-deficient cells upon incubation with BM or MMS, as well as accumulation of unrepaired DNA lesions after recovery from MMS-damage, (iii) increased number of spontaneous AP sites and spontaneous mutation frequencies in *gapA* mutants versus wild-type isogenic strains and (iv) interaction of GAPDH with SSB and proteins of the BER pathway, namely UDG and Endo IV.

The filamentous growth of *gapA* null mutants and GAPDH-silenced cells treated with BM or MMS is a sign of the SOS response. As stated above, AP sites are BER intermediates formed in MMS-treated bacterial cells after excision of methylated bases by DNA glycosylases. These abasic sites are also directly generated by the action of BM on DNA. There is evidence that accumulation of AP sites triggers the SOS response. When the repair of alkylated bases by the BER system is insufficient, other pathways connected to the SOS response, like homologous recombination, contribute to DNA repair (Janion et al., 2003). Thus, filamentous growth of GAPDH-deficient cells is consistent with the inability of these cells to efficiently repair the AP sites generated by either genotoxic agent. Indeed, results from alkaline DNA degradation assays point to more unrepaired lesions in the genomic DNA of the Δ gapA mutant than in the parental strain after 3-h recovery from extensive MMS damage.

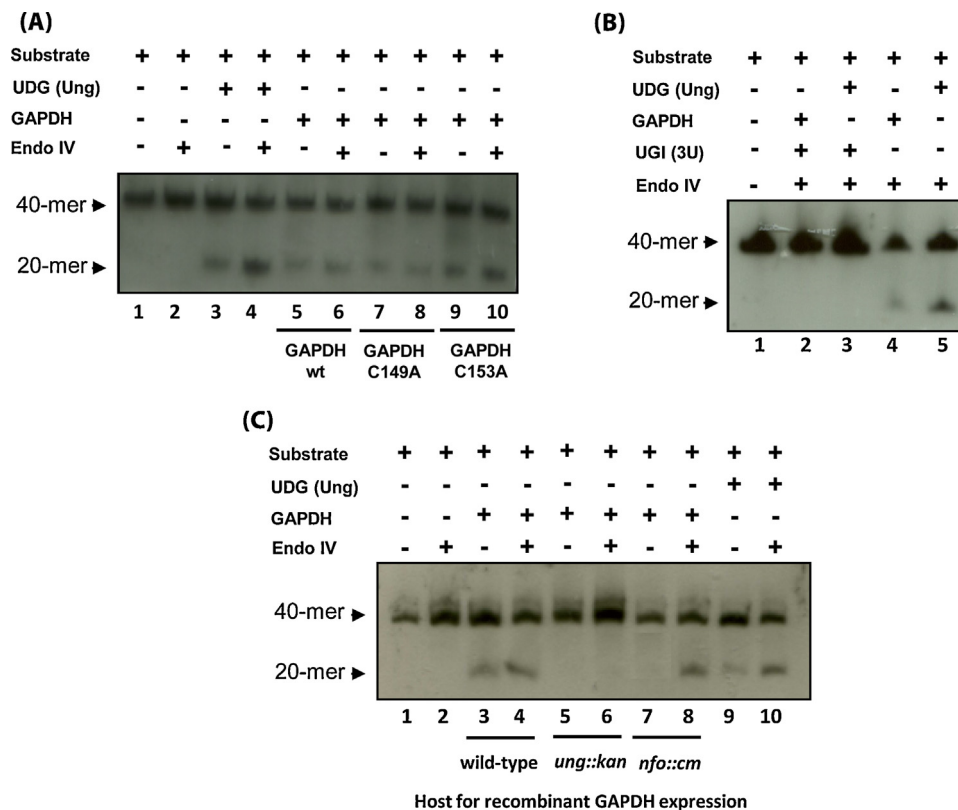


Fig. 7. GAPDH does not display UDG activity. (A) Analysis of the UDG activity of purified GAPDH. The 40-mer duplex DNA substrate containing a U-G mismatch (U19.G) was incubated with commercial *E. coli* UDG (Ung) as a positive control of the UDG activity (lanes 3 and 4) or with 10 μ g of the indicated purified GAPDH variants (lanes 5–10). These GAPDH variants were expressed and purified from strain MC4100 Δ *gapA* using the GST fusion system recognition sites for factor X cleavage. When indicated, commercial Endo IV was added to the reaction mixture. Shown are the controls, substrate alone (lane 1) or incubated only with Endo IV (lane 2). The arrows indicate positions of the 40-mer substrate and the 20-mer product. (B) Inhibition of the GAPDH-associated UDG activity by Ugi. The substrate was incubated with commercial UDG (lanes 3 and 5) or with 10 μ g of purified GAPDH (lanes 2 and 4) in the absence or presence of the inhibitor Ugi. Commercial Endo IV was added to all reactions. The control corresponding to the substrate alone (lane 1) is shown. (C) GAPDH-associated UDG activity is due to accompanying chromosome-encoded Ung. The substrate was incubated with 10 μ g of wild-type GAPDH expressed and purified from strain MG1655 or from the mutant strains MG1655 *ung::kan* (deficient in UDG) and MG1655 *nfo::cm* (deficient in Endo IV). When indicated, commercial Endo IV was added to the reaction mixture. The control corresponding to the substrate alone (lane 1) as well as the positive reaction control with commercial UDG (lanes 9 and 10) are shown.

Repair of AP sites depends on the activity of AP-endonucleases. *E. coli* has two AP-endonucleases, Exo III and Endo IV. Exo III is constitutively expressed while Endo IV is inducible by oxidative stress. These enzymes are structurally unrelated and belong to different families of AP-endonucleases. The Exo III family members are present in all kingdoms, whereas Endo IV members are expressed only in lower organisms (Daley et al., 2010). As stated above, human GAPDH interacts with and modulates the activity of APE1, which displays 52% similarity with *E. coli* Exo III. Results of our pull-down experiments indicate that *E. coli* GAPDH interacts with Endo IV but not with Exo III. Human APE1 has 39 amino acid residues at the N-terminus that are not present in Exo III. This difference may affect protein interactions. As Endo IV does not undergo redox reaction as does APE1, the role of GAPDH in repairing oxidized Endo IV seems not plausible. Interaction between GAPDH and Endo IV does not imply that GAPDH modulates Endo IV, but may serve to assist other DNA repairing activities.

Although Exo III is considered the major AP-endonuclease in *E. coli* (Cunningham et al., 1986), several studies show relevant contribution of Endo IV in repairing DNA damage caused by some genotoxic agents. In this regard, *nfo* mutants are hypersensitive to BM and *t*-butyl hydroperoxide, and mildly hypersensitive to MMS and mitomycin (Cunningham et al., 1986). Endo IV also significantly contributes to the repair of AP sites following endogenous DNA damage, which occurs continuously in nature. AP sites are both mutagenic and cytotoxic, thus DNA repair mechanisms are essential to correct replication and transcription. Studies performed with

single *nfo* or *xth* mutants showed a greater contribution of Endo IV than Exo III in preventing transcriptional mutations caused by the formation of AP sites (Clauson et al., 2010). Here, we provide evidence that the ability to repair spontaneous AP sites is reduced in GAPDH-deficient cells. During aerobic exponential growth, the *gapA* null mutant displays a 1.5-fold increase in spontaneous AP sites when compared to the wild-type strain. Consistently, the frequency of spontaneous mutation was higher in this mutant. The about 4.7-fold increase in the spontaneous mutation frequency estimated in this study for strain MC4100 Δ *gapA* is similar to the values reported for *E. coli* *ung* or *nfo* single mutants, which were 5.1- and 4.6-fold, respectively (Barthi and Varshney, 2010; Sikora et al., 2010).

One of the first moonlighting functions associated with human GAPDH was its activity as uracil-DNA glycosylase (Meyer-Siegler et al., 1991). Later studies conducted to purify and characterize two different human uracil-DNA glycosylases (UDG1 and UDG2) did not detect such activity associated with human GAPDH. Instead, this activity was attributed to contaminating proteins present in the commercial erythrocyte GAPDH sample, probably UDG1 or UDG2 (Caradonna et al., 1996). Likewise, our results rule out UDG activity in *E. coli* GAPDH. The uracil-removing activity detected with our GAPDH preparations (either the wild-type protein or the Cys mutated variants) may be attributed to the chromosome-encoded UDG (*ung* gene product) as this activity was no longer identified when the recombinant GAPDH protein was expressed in an *ung* mutant background. Overall, these results indicate that GAPDH

is strongly associated with UDG enzymes both in humans and in bacteria. On the other hand, the two commercial sources of *E. coli* UDG used here (Sigma or New England Biolabs) contained an AP-endonuclease accompanying activity, thus indicating a strong binding affinity between the enzyme that removes uracil from DNA and the nuclease that repairs the AP site. Based on our results, this AP-endonuclease could be Endo IV.

The reported interaction between GAPDH and Gph suggested involvement of GAPDH in protein complexes that repair BM-induced DNA lesions (Ferreira et al., 2013). Our results here confirm that *E. coli* GAPDH is required for efficient repair of AP sites through the BER pathway. In this context, its interaction with Endo IV and UDG indicates that GAPDH is a component of protein complexes dedicated to the maintenance of genomic DNA integrity.

Since Endo IV displays additional roles not shared by Exo III, the interaction of GAPDH with Endo IV implicates GAPDH in other DNA repair pathways like the nucleotide incision repair (NIR) pathway. Endo IV contributes to this pathway by processing a broad range of DNA lesions like those caused by ionizing radiation damage on cytosine residues under either oxidative or anoxic conditions (Ishchenko et al., 2004; Daviet et al., 2007). Our study has also shown interaction of GAPDH with SSB, an essential protein that interacts with single-stranded DNA preventing the formation of secondary structures and its degradation by nucleases (Greipel et al., 1987; Dabrowski et al., 2002; Furukohri et al., 2012). SSB has a crucial role in the recruitment of proteins involved in DNA replication and repair, such as homologous recombination and SOS response (Furukohri et al., 2012). In fact, *E. coli* *ssb* mutants are highly sensitive to several alkylating agents, including MMS (Greenberg et al., 1974, 1975). Thus, interaction of GAPDH with SSB may recruit GAPDH to the DNA repair sites. The filamentous growth of GAPDH-deficient cells and the interaction of GAPDH with SSB point toward GAPDH as a component of protein complexes involved in DNA repair pathways activated under profuse DNA damage.

The functional role of GAPDH in DNA repair is not yet known. As a component of protein complexes, GAPDH may act as a scaffold protein or may have a role in modulating the activity of other protein components.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocel.2015.01.008>.

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