Protein interaction studies point to new functions for *Escherichia coli* glyceraldehyde-3-phosphate dehydrogenase

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Abstract

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is considered a multifunctional protein with defined functions in numerous mammalian cellular processes. GAPDH functional diversity depends on various factors such as covalent modifications, subcellular localization, oligomeric state and intracellular concentration of substrates or ligands, as well as protein-protein interactions. In bacteria, alternative GAPDH functions have been associated with its extracellular location in pathogens or probiotics. In this study, new intracellular functions of *E. coli* GAPDH were investigated following a proteomic approach aimed at identifying interacting partners using *in vivo* formaldehyde cross-linking followed by mass spectrometry. The identified proteins were involved in metabolic processes, protein synthesis and folding or DNA repair. Some interacting proteins were also identified in immunopurification experiments in the absence of cross-linking. Pull-down experiments and overlay immunoblotting were performed to further characterize the interaction with phosphoglycolate phosphatase (Gph). This enzyme is involved in the metabolism of 2-phosphoglycolate formed in the DNA repair of 3'-phosphoglycolate ends generated by bleomycin damage. We show that interaction between Gph and GAPDH increases in cells challenged with bleomycin, suggesting involvement of GAPDH in cellular processes linked to DNA repair mechanisms.

Keywords: moonlighting proteins; glyceraldehyde-3-phosphate dehydrogenase; protein-protein interactions; phosphoglycolate phosphatase; DNA repair; *Escherichia coli*

58 **Abbreviations**: CAA, casein acid hydrolysate CAA; CFUs, colony forming units; FA,
59 formaldehyde; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gph,
60 phosphoglycolate phosphatase; GST, glutathione-S-transferase; MS, mass spectrometry;
61 NBRP, National BioResource Project; PVDF, polyvinylidene difluoride.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a key enzyme in the glycolytic pathway, is not merely a classical metabolic protein, but is also considered a multifunctional protein with defined functions in numerous mammalian cellular processes (reviewed in Sirover, 1999, 2005, 2011). This protein is involved in transcriptional and post-transcriptional gene regulation, chromatin structure, intracellular membrane trafficking, DNA replication and DNA repair processes (Sirover, 2011). Each new function requires GAPDH association into specific protein complexes. The functional diversity of GAPDH depends on various factors, such as covalent modifications, subcellular localization, oligomeric state, or intracellular concentration of substrates (Jeffery, 2004; Sirover, 2005, 2011). In this context, it is important to consider the great diversity of post-translational modifications described for GAPDH, which may underpin its multifunctional activity (Sirover, 2011). Many of these modifications are associated with various types of stress (Cabiscol and Ros, 2006; Colussi et al., 2000; Eaton et al., 2002).

In bacteria, alternative GAPDH functions have been associated with its extracellular location in pathogens. GAPDH is one of the housekeeping proteins that are secreted and exposed on the bacterial surface, enabling the pathogens to colonize and persist in the host (Pancholi and Chhatwal, 2003). Several reports on different gram-positive pathogens have demonstrated the ability of extracellular GAPDH to interact with various host components such as transferrin, plasminogen, extracellular matrix components or the urokinase plasminogen activator receptor (Jin et al., 2005; Schaumburg et al., 2004; Seifert et al., 2003). In the case of the gram-negative pathogens enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), our group showed that secreted GAPDH is able to

interact with human plasminogen and fibrinogen, and remains associated with Caco-2 cells upon infection (Egea et al., 2007). Extracellular GAPDH can also act as a target for oxidation and therefore may protect bacteria against host oxidative response (Aguilera et al., 2009). In recent years, GAPDH has been identified in the secretome of probiotic strains (Aguilera et al., 2012; Sanchez et al., 2009a, 2009b). Extracellular GAPDH may confer these strains an advantage in gut colonization with respect to other components of the intestinal microbiota. Besides the role of secreted GAPDH in host colonization processes, no studies of new GAPDH intracellular functions have been described so far in bacteria.

In the area of systems biology, the identification of protein-protein interactions within cells is of prime importance to understand biological processes at the molecular level. Several groups have performed large-scale analyses using *E. coli* to identify interactions between proteins (Arifuzzaman et al., 2006; Butland et al., 2005). These studies, based on tagged protein baits that allow affinity purification of the interacting proteins, have shown that GAPDH interacts with a range of proteins, including metabolic enzymes and proteins involved in transcription or protein synthesis, suggesting the potential role of *E. coli* GAPDH in some cellular processes similar to those described in humans.

When using these affinity-based methods, transient interactions or weakly binding proteins can be lost during washing procedures. As an alternative, *in vivo* chemical cross-linking experiments can be performed to covalently fix interaction partners in living cells, thus preventing the loss of specific components of a protein complex prior to purification (Sinz, 2010). Although formaldehyde is widely used as a cross-linker in chromatin immunoprecipitation experiments, the number of studies which have used this reagent in combination with mass spectrometry (MS) to investigate protein-protein interaction is

limited (Sinz, 2010). Examples of this approach are the identification of new proteins interacting with a constitutive active form of M-Ras (Vasilescu et al., 2004) or the identification of glutamate dehydrogenase as a protein that interacts with the regulator GltC in *Bacillus subtilis* (Herberg et al., 2007).

To explore new GAPDH functions in *E. coli*, in this study we conducted *in vivo* formaldehyde cross-linking experiments followed by MS to identify proteins interacting with V5-tagged GAPDH in *E. coli*. We also characterized interaction with phosphoglycolate phosphatase (Gph), an enzyme involved in the metabolism of 2-phosphoglycolate, a compound formed in the DNA repair of 3'-phosphoglycolate ends generated by bleomycin damage (Pellicer et al., 2003).

2. Materials and methods

2.1. Bacterial strains and growth conditions

The genotypes and sources of the *E. coli* strains used in this study are as follows. Strain MC4100 (F^- *araD Δ(argF-lac) rpsL*(Str^r) *relA fihD deoC ptsF rbs*) (Casadaban, 1976) was the source of chromosomal DNA used as a template for PCR amplification of gene *gapA*. The mutant strain JA210 (*gph::Kan*) was derived from MC4100 (Pellicer et al., 2003). Strain TOP10 (F^- Δ*mrcA* (*mrr-hsdRMS-mcrBC*) ϕ*80lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu)7697 galU galK rpsL*(Str^r) *endA1 nupG*) was used to express recombinant GAPDH-V5 (Invitrogen). Strain BL21(DE3) (F^- *ompT hsd*(r_B^- m_B^-) *gal*) was used as a host to express GST-tagged proteins (Amershan Pharmacia). Strain AG1 (ME5053) (*recA1*

endA1 gyrA96 thi-1 hsdR17(r_K^- m_K^-) *supE44 realA1*) was the host for recombinant pCA24N plasmids expressing His-tagged proteins (NBRP, National BioResource Project). Bacterial cells were routinely grown at 37ºC in Luria-Bertani broth (LB). In bleomycin challenge experiments, cells were grown in minimal medium with 0.5% casein acid hydrolysate (CAA) as carbon source (Pellicer et al., 2003). Growth was monitored by measuring the optical density at 600 nm (OD_{600}). When required, tetracycline (12.5 μg/ml), chloramphenicol (30 μg/ml) or ampicillin (100 μg/ml) was added to the medium.

2.2. Recombinant DNA techniques and site-directed mutagenesis

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 Rusell, 2001). DNA fragments were amplified by PCR using *E. coli* chromosomal DNA as a template. PCR reactions were performed with Taq DNA polymerase or *pfu* DNA polymerase under standard conditions. DNA sequencing was carried out with an automated ABI 377 DNA sequencer and fluorescent dye termination methods.

2.3. Cloning of recombinant V5-tagged GAPDH

To construct recombinant GAPDH-V5, the pBAD TOPO® TA Expression Kit (Invitrogene) was used. This system provides a highly efficient, one-step cloning strategy for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector for regulated expression in *E. coli*. Expression is driven by the *araBAD* promoter, thus

induction of the cloned gene is achieved by the addition of L-arabinose to the culture medium. The *gapA* gene from strain MC4100 was amplified by PCR with primers gapA-pBAD-fw (TAGGGTGGAATAT**ATG**ACTATCAAAGTAGG) and gapA-pBAD-rv (TTTGGA GATGTGAGCGATCAGG). To express GAPDH with its native N-terminal end (without the N-terminal leader), the forward primer was designed with an in-frame stop codon (TAG, underlined) at the 5'-end followed by the native *gapA* sequence including the ribosome binding site and the start codon (in bold). The *gapA* stop codon was removed from the reverse primer to allow expression of GAPDH fused at its C-terminal end to the V5 epitope. The PCR fragment was cloned into plasmid pBAD-TOPO (Invitrogen) and after transformation of strain TOP10, recombinant colonies were selected on LB- ampicillin plates. Plasmid DNA was sequenced to ensure that the fragment was inserted in the correct orientation and that no mutations were introduced. The recombinant plasmid was named pBAD-GapA.

2.4. In vivo cross-linking with formaldehyde

Cells of strain TOP10 bearing the recombinant plasmid pBAD-GapA were grown at 37ºC in 10 ml of LB-ampicillin until the culture reached an OD_{600} of 0.5. At this point L-arabinose was added at a final concentration of 0.02% to induce the expression of GAPDH-V5. After a 3 h induction, formaldehyde was added at a final concentration of 1%. Formaldehyde cross-linking was allowed to proceed for 20 min at 37ºC. To stop the cross-linking reaction, glycine was added at a final concentration of 0.5 M and after 5 min at room temperature, bacterial cells were harvested by centrifugation, washed twice with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), and resuspended in 0.4 ml of PBS containing a cocktail of protease inhibitors (Roche

Diagnostic). Cell extracts were obtained by sonic disruption of bacterial cells followed by centrifugation to remove cell debris. Protein concentration was determined using the method described by Lowry et al. (1951) with bovine serum as a standard.

2.5. Immunoprecipitation of recombinant GAPDH-V5

Purification of GAPDH-V5 cross-linked complexes was performed by immunoprecipitation using anti-V5 agarose beads (Sigma-Aldrich, Germany). To reduce unspecific interactions, the binding and washing steps were carried out under astringent conditions with RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 0.14 M NaCl, 0.01 M Tris-HCl, pH 8.0). Cell extracts were precleared with protein G-agarose beads for 10 min at 4ºC. Precleared cell extracts containing 1.4 mg of protein were adjusted to a final volume of 0.8 ml with RIPA buffer and incubated with 250 µl of anti-V5 beads for 90 min at 4ºC in an orbital shaker. After five washes with RIPA buffer and two additional washes with TBS buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.6), the bound complexes were eluted with 0.1 M glycine (pH 2.5) for 15 min at 37ºC and neutralized with a saturated Tris base solution. Protein from the eluted fractions was precipitated with acetone (at room temperature to minimize SDS precipitation), rinsed three times in acetone, and resuspended in loading buffer. Samples were then heated for 30 min at 95ºC to reverse the formaldehyde cross-links. Proteins were separated on a 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and visualized by Sypro® Ruby staining.

Immunoprecipitation experiments with anti-V5 agarose beads were also carried out to identify proteins that interact with GAPDH without previous cross-linking. In this case, precleared cell extracts containing 1.4 mg of protein were adjusted to a final volume of 0.8

ml with PBS buffer and incubated with anti-V5 beads as described above. After five washes with PBS buffer, proteins were eluted with 0.1 M glycine (pH 2.5) for 15 min at 37ºC, neutralized and precipitated by incubation on ice with 10% trichloroacetic acid (TCA). The protein pellet was washed in 90% (v/v) ice-cold acetone, air-dried and suspended in rehydration buffer (9M urea, 4% CHAPS, 50 mM dithiothreitol, 0.5% immobilized pH gradient buffer and traces of bromophenol blue) and processed for two-dimensional gel electrophoresis.

2.6. Two-dimensional gel electrophoresis

2D gel electrophoresis was performed using the Protean IEF-Cell (Bio-Rad). Appropriate volumes of protein samples (5 μg of purified recombinant GAPDH) were diluted in 125 μl of rehydration buffer (9 M urea, 4% CHAPS, 50 mM dithiothreitol, 0.5% immobilized pH gradient buffer and traces of bromophenol blue). Isoelectric focusing was performed in immobilized pH 3-10 or pH 5-8 gradient strips (BioRad). Second dimension SDS-PAGE was performed on 12.5% acrylamide gels that were processed for immunoblotting analysis. Parallel gels were silver-stained to visualize total protein.

2.7. Mass spectrometry of proteins

Following SDS-PAGE, protein bands of interest were excised from the gel and sequentially washed with 25 mM ammonium bicarbonate and acetonitrile. These samples were then reduced (10 mM DTT, 30 min at 56ºC), alkylated (55 mM iodoacetamide, 15 min at 21ºC) and digested overnight with tripsin (Tripsin Gold, Promega) at 37ºC in an automatic Investigator ProGest robot (Genomic Solutions). Peptides were extracted with

10% formic acid and analysed by combined liquid chromatography / tandem mass spectrometry (Cap-LC-nano-ESI-Q-TOF) (CapLC, Micromass-Waters) at the Barcelona Science Park (PCB) Proteomics Platform. Data were generated in PKL file format and submitted to a MASCOT server for database searching.

Protein spots from 2D gels were analysed using the liquid chromatograph nanoAcquity (Waters) coupled to a mass spectrometer (OrbitrapVelos, Thermo Scientific). Raw data were obtained with Thermo Xcalibur (v.2.1.0.1140) and used for database searching with the Proteome Discoverer software package (v.1.2.0.124).

2.8. Expression and purification of recombinant proteins

Recombinant GAPDH and Gph were expressed and purified using the Glutathione-S-transferase (GST) gene fusion system with recognition sites for factor Xa cleavage as described in detail previously (Egea et al., 2007; Pellicer et al., 2003).

When indicated, Gph was expressed as His_6-Gph from the ASKA clone JW3348 (NBRP, *E. coli* Strain National BioResource Project) and purified under native conditions with Ni^{2+}-nitrilotriacetic acid (NTA) resin (Qiagen), as described elsewhere (Kitagawa et al., 2005). The same procedure was applied for expression and purification of the ATP synthase β subunit (His_6-AtpD) and the ATP synthase α subunit (His_6-AtpA) from the ASKA clones JW3710 and JW3712, respectively. Due to the low level expression of soluble His_6-AtpD, purification of this protein was achieved from a 100 ml culture of strain AG1 after overnight induction with 0.1 mM IPTG at 20ºC. Cell pellets were suspended in 1 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole, and sonicated on ice. The cell lysate was centrifuged at 15000 *g* and the

supernatant incubated with 0.1 ml of Ni^{2+}-NTA resin for 1 h at 4ºC with gentle shaking. After loading the mixture into a column, the resin was washed with the same buffer but containing 20 mM imidazole. Recombinant proteins were eluted with 0.1 ml of elution buffer containing 300 mM imidazole, followed by SDS-PAGE and immunoblotting analysis.

2.9. Immunoblotting analysis

For Western blot analysis, protein samples were separated on 10% SDS-PAGE and transferred to a HyBond-P polyvinylidene difluoride (PVDF) membrane by using a Bio-Rad MiniTransblot apparatus. The membrane was blocked in PBS-0.05% Tween-20 and 5% skimmed milk (blocking solution) for 1 h at room temperature, incubated with primary specific antibodies against GAPDH (Egea et al., 2007) (1:5,000 dilution in blocking solution) for 16 h at 4°C, washed four times with PBS-0.05% Tween-20 and incubated with the secondary antibody (donkey anti-rabbit immunoglobulin horseradish peroxidase-linked, diluted 1:15,000 in blocking solution). Incubation with the secondary antibody was omitted when anti-V5 (Invitrogen; 1:15,000 dilution) or anti-GST (GenScript; 1:5,000 dilution) horseradish peroxidase-linked antibodies were used as primary antibody. The protein-antibody complex was visualized using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech).

Binding of GAPDH to Gph was analysed by Far-Western assays (overlay immunoblotting). In this case, purified GAPDH was subjected to 2D gel electrophoresis as described above. After being blocked overnight with 1% gelatin in TBS buffer, the membrane was incubated with purified Gph (5 μg/ml) diluted in the same blocking buffer

for 3 hours and then washed four times in TBS-0.05% Tween-20. To visualize interaction of GAPDH with Gph, the membrane was incubated with anti-Gph specific antibodies (1:5,000 dilution TBS-1% gelatin-0.05% Tween-20) for 16 h at 4ºC, and processed as described above using the ECL Western blotting kit to visualize the reactive spots. The incubation step with purified Gph was omitted as negative control.

The anti-Gph antibodies used in this study were purified by affinity chromatography. For this purpose, purified Gph was covalently linked to Ultralink^{TM} Immobilized DADPA on 3 M Emphase Biosupport medium AB1 gel (Pierce) using water-soluble 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide-HCl. To purify the anti-Gph immunoglobulin, 3 ml of the globulin fraction partially purified from the Gph-antisera (Pellicer et al., 2003) was applied to the DADPA-Gph affinity column and the bound antibodies were eluted with 0.1 M glycine buffer (pH 2.5). Collected fractions were neutralized with Tris solution (pH 10) and dialysed overnight against PBS.

2.10. Bleomycin cell treatment

Cells of strain JA210 bearing plasmid pGEX-Gph (Pellicer et al., 2003) were grown aerobically in minimal medium with 0.5% CAA to mid-exponential phase (OD_{600} of 0.5). After a 2 hour induction of GST-Gph expression with 0.5 mM IPTG, the culture was treated with 8 μg/ml bleomycin in the presence of 50 μM $FeSO_4$. At different times, aliquots of the culture were collected, washed twice with minimal medium and processed to obtain the cell extracts. Interaction between Gph and GAPDH was evaluated by pull-down experiments followed by Western blot analysis with antibodies against GAPDH. Survival was estimated by diluting cells in the same medium, followed by plating on LB-ampicillin

plates to determine the number of colony forming units (CFUs). Bleomycin sulphate (Almirall Prodesfarma, Barcelona, Spain) was freshly prepared with 50 mM phosphate buffer (pH 7.5). $FeSO_4$ solutions were prepared immediately before use.

3. Results and discussion

3.1. Immunoaffinity purification of proteins cross-linked to GAPDH-V5 and their identification by mass spectrometry

Cultures of TOP10 cells expressing V5-tagged GAPDH were treated with formaldehyde and the GAPDH-V5 containing complexes were analysed in the cell extracts by Western blotting with either anti-V5 or anti-GAPDH antibodies. As control, TOP10 cells without the recombinant pBAD-GapA plasmid were processed in parallel. As shown in Fig. 1A, protein bands ranging in size from 70 kDa to 160 kDa were only detected above recombinant GAPDH-V5 (37 kDa) in cells treated with the cross-linker. The most apparent bands corresponded to high molecular protein complexes in accordance with the native GAPDH tetramer structure. The presence of protein complexes with a molecular mass lower than 160 kDa indicates that other oligomeric or monomeric GAPDH-V5 forms were being cross-linked with *E. coli* proteins. To set up the conditions for cross-linking reversal, cell extracts were incubated at 65ºC or 95ºC in loading buffer and at the indicated times, reversion of the cross-links was analysed by Western blotting with anti-V5 antibodies (Fig. 1B). The high molecular mass complexes produced by formaldehyde treatment were no longer detected after 30 min incubation at 95ºC. These conditions were selected for further experiments.

In order to identify the proteins that were cross-linked to GAPDH-V5, precleared cell extracts obtained from TOP10 (pBAD-GapA) cells challenged with formaldehyde were incubated with anti-V5 beads. After astringent washing steps, bound material was eluted as described in section 2.5. Cell extracts obtained from non formaldehyde-treated TOP10 (pBAD-GapA) cells were processed in parallel. Purified complexes were concentrated by acetone precipitation, resuspended in loading buffer and processed to reverse formaldehyde cross-links. Proteins were separated by SDS-PAGE. Staining with Sypro® Ruby revealed several proteins in the cross-linked sample that were not visible in the lane corresponding to non-treated cells (Fig. 2). These differential proteins were selected for further analysis. In the absence of formaldehyde treatment, co-purification of native GAPDH (band 2) with recombinant GAPDH-V5 (band 3) under astringent conditions (Fig. 2, - FA lane, Table 1) indicated a strong interaction of GAPDH monomers compatible with the stable tetrameric structure reported for this protein.

Twelve major bands were excised from the cross-linked sample (Fig. 2, + FA lane) and digested with trypsin. Peptides were extracted and analysed by combined liquid chromatography / tandem mass spectrometry (Cap-LC-nano-ESI-Q-TOF). Database searching in MASCOT resulted in the identification of 16 proteins, each of which matched to at least 3 unique peptide sequences (Table 1). Among the identified proteins, there were several metabolic enzymes, chaperones and factors involved in protein synthesis.

Four proteins (trigger factor, DnaK, Gph and aldehyde dehydrogenase (AldA)) also appeared as candidates for interaction with GAPDH in other studies of *E. coli* or in databases such as DIP (database of interacting proteins) or IntAct. The chaperones DnaK (P0A6Y8) and trigger factor (P0A850) were identified in large-scale analyses (Arifuzzaman et al., 2006; Butland et al., 2005). Trigger factor interacts with the large

ribosomal subunit near the peptide exit channel and binds to nascent polypeptides to assist cotranslational protein folding. Therefore, interaction of GAPDH-V5 with this chaperone is likely to occur during the synthesis of the recombinant polypeptide. In addition, trigger factor has been shown to prevent aggregation and promote refolding of denatured GAPDH *in vitro* (Huang et al., 2000). GAPDH has also been identified as a substrate of DnaK (Deuerling et al., 2003). Both trigger factor and DnaK share a common substrate pool *in vivo* and cooperate to ensure proper folding of the cytosolic proteins (Deuerling et al., 1999, 2003). Thus, interaction between the synthesized recombinant protein and these chaperones is expected to occur in cells grown at 37ºC.

GAPDH was identified as a putative interacting partner of Gph (P32662) in the large-scale study performed by Arifuzzaman et al. (2006). In this case, the interaction was observed with His_6-Gph used as the bait in pull-down experiments. Regarding the interaction with AldA (P25553), information available on the IntAct database http://www.ebi.ac.uk/ intact/pages/interactions/ interactions) corroborates our results. Both GAPDH and AldA were identified together with L-asparaginase-2 (P00805) as partners of a protein complex resolved by native-PAGE.

The other proteins identified in this study as putative partners of GAPDH interactions (Table 1) have not been experimentally isolated in previous studies. However, some of them are predicted to be functional partners of GAPDH in the STRING interaction network (http://string-db.org/newstring_cgi/ show_network_ section.pl). Enolase and transketolase belong to this group. Other proteins may establish weak or transient interactions with GAPDH, being captured in our experimental conditions by formaldehyde cross-linking. Alternatively, some interactions may be indirect through association of these proteins as partners of multiprotein complexes.

3.2. Identification of proteins that co-purify with GAPDH-V5 in the absence of cross-linking

Immunoprecipitation experiments with anti-V5 agarose beads were also carried out to identify proteins that interact with GAPDH without previous cross-linking. Precleared cell extracts were processed as described in section 2.5. Purified proteins were concentrated by TCA precipitation, resuspended in rehydration buffer and processed for 2D gel electrophoresis. In addition to the dominant spots corresponding to recombinant GAPDH-V5 and native GAPDH forms, staining with Sypro® Ruby also revealed other proteins that co-purified with GAPDH-V5 under these conditions (Fig. 2B). An immunoprecipitation experiment starting with precleared cell extracts of TOP10 cells was performed as control. In the absence of a V5-tagged protein, no apparent spots were visible (not shown), thus ruling out co-purification of these proteins through interactions with the affinity matrix.

Ten spots were excised from the 2D gel (Fig. 2B) and analysed by LC-MS/MS, in this case using a high sensitivity OrbitrapVelos mass spectrometer that enables identification of proteins even at very low amounts. Results are summarized in Table 2. Among these proteins, the ATP synthase β subunit (AtpD) and the porin OmpC, both displaying high scores in this analysis, were also identified as proteins interacting with GAPDH after formaldehyde cross-linking (Table 1). Regarding the ATP synthase α subunit (AtpA) and LpdA (both in spot 1), although these proteins were not listed in Table 1, the MS analysis performed in the cross-linking experiment revealed in both cases two peptides matching these sequences. Another protein found here to co-purify with GAPDH was GatY (30.8 kDa), the catalytic subunit of D-tagatose-1,6-bisphosphate aldolase (Brinkkötter et al., 2002). Large-scale proteomic studies performed by Burtland et al. (2005) identified the

regulatory subunit GatZ as an interacting partner of GAPDH. Neither GatY nor GatZ were identified as interacting with GAPDH in the cross-linking experiment. However, it should be noted that in this experiment, only proteins specifically present in the FA-treated sample were excised and analysed by MS. In this sense, GatY should correspond to one of the non-excised protein bands with a molecular mass around 30 kDa, also visible in the control sample (Fig. 1A lane –FA versus +FA).

The protein displaying the highest score in this analysis was AtpD, with 40 peptides identified that covered 85.87% of the sequence (Table 2). This ATP synthase subunit interacts with the α subunit (AtpA) to form the catalytic domain for ATP synthesis. AtpA also co-purified with GAPDH-V5, although the MS analysis yielded a lower score. In this case, 29 peptides were identified that covered 61.79 % of the sequence (Table 2).

To validate interaction of GAPDH with both ATP synthase subunits, pull-down experiments with His_6-tagged AtpD and AtpA proteins were performed. Recombinant His_6-AtpD and His_6-AtpA were expressed and purified from the ASKA clones JW3710 and JW3712, respectively, as described in section 2.7. Immunoblotting of the column fractions with anti-GAPDH antibodies indicated that a fraction of GAPDH was bound and co-eluted with either His_6-AtpD or His_6-AtpA (Fig. 3). In a control experiment, the cell extract obtained from the host strain AG1 was directly applied to the Ni^{2+}-NTA column and processed in parallel as a control to confirm that the Ni^{2+}-NTA resin did not bind GAPDH to any extent (Fig. 3, right panel). It is noteworthy that even at lower bait protein concentration the GAPDH fraction recovered in these experiments was higher in the case of His_6-AtpD. This suggests that co-purification and binding of GAPDH to AtpA may be

412 indirect through its interaction with the AtpD subunit. Alternatively, association of the
413 AtpD and AtpA may be required to provide the interaction domain with GAPDH.
414 It should be noted that in addition to AtpA, spot 1 contained other ATP-binding
415 proteins such as AraG and RbsA. Abundance of these proteins in the spot should be very
416 low given the low score and number of sequenced peptides (Table 2). Their identification
417 may be attributed to the high sensitivity achieved with the analysis through use of the
418 OrbitrapVelos mass spectrometer. All the proteins identified in spot 1 displayed similar
419 molecular mass and pI, which is compatible with their inclusion in a single spot (Table 2).
420 The fact that out of these five proteins, three contained ATP-binding domains suggests that
421 GAPDH may display affinity for such domains. These kinds of interaction may point to a
422 regulatory role of GAPDH in energy-dependent processes. One such process is cell
423 motility. In this context, GAPDH has been suggested to play an important role in the
424 motility of the halotolerant alga *Dunaliella salina*. In this alga, GAPDH was found in the
425 flagellar proteome; in addition, silencing of GAPDH expression by RNAi led to reduced
426 motility (Jia et al., 2009).
427 A relationship between GAPDH and cellular processes involved in energy production
428 and conversion has been observed in *Streptococcus pyogenes* (Jin et al., 2011). Besides its
429 cytoplasmic location, surface export of GAPDH is essential for *Streptococcus* virulence.
430 Mutants unable to export this protein, and thus expressing higher intracellular GAPDH
431 levels, displayed an 8-fold increase in the intracellular ATP concentration with respect to
432 the wild-type strain. Moreover, this study revealed that intracellular levels of GAPDH in
433 some way control the expression of certain genes (Jin et al., 2011). For instance, microarray
434 analysis of this mutant revealed up-regulation of genes encoding proton-translocating
435 ATPases. In this context, the interaction of GAPDH with ATP synthase subunits identified

here suggests that there are additional mechanisms in the regulatory role of GAPDH in ATP-dependent processes.

3.3. Analysis of GAPDH interaction with Gph

Among the proteins identified as potential GAPDH interacting partners, we found the interaction with Gph to be of special interest due to its physiological role in processes linked to DNA repair. Our group showed for the first time in bacteria that this housekeeping enzyme is involved in the dissimilation of the intracellular 2-phosphoglycolate formed in the DNA repair of 3'-phosphoglycolate ends (Pellicer et al., 2003). These kinds of DNA strand break are caused by bleomycin (Povirk, 1996). In mammals, involvement of GAPDH in DNA repair processes has been well-documented (Sirover, 2005; Azam et al., 2008); however no reports on a similar function were available for prokaryotic GAPDH.

First, we aimed to determine whether GAPDH copurified with recombinant Gph. To this end, cell extracts of strain BL21 bearing plasmid pGEX-Gph, and therefore expressing GST-Gph (Pellicer et al., 2003), were applied to a glutathione-sepharose 4B resin. Cell extracts expressing the tag protein GST were processed in parallel as control. After extensive washing, elution of GST or GST-Gph was performed with glutathione elution buffer. Western blot analysis of the eluted fractions indicated that GAPDH co-purified with GST-Gph but not with GST (Fig. 4A). In a parallel experiment, elution of Gph from the affinity resin was achieved by incubation with factor Xa. Western blot analysis confirmed co-elution of GAPDH (Fig. 4B). In the eluted fractions, in addition to Gph (27 kDa), two other proteins were visible. The 34 kDa protein is one of the factor Xa subunits. The 35 kDa protein was excised from the gel and identified as GAPDH by MS. Moreover, pull-

down experiments using His_6-Gph expressed in strain AG1 from the ASKA clone JW3348 as bait confirmed co-purification of GAPDH with Gph (not shown).

Overall these results confirmed interaction of GAPDH with Gph. However, when purified GAPDH was applied to an immobilized GST-Gph column, GAPDH was scarcely retained (not shown). Most of the GAPDH protein appeared in the flow-through fraction, suggesting that interaction between these proteins may either depend on other cellular factors (proteins or ligands) or on post-translational modifications of these proteins. The presence of multiple covalently modified forms of GAPDH (Aguilera et al., 2009; Egea et al., 2007) prompted us to analyse whether the Gph binding activity is specifically linked to one of these forms. This was determined by Far-Western experiments in which purified GAPDH was subjected to 2D gel electrophoresis. Once electroblotted, the membrane was reacted with purified Gph followed by incubation with specific anti-Gph antibodies. Of the multiple GAPDH spots, only one was visualized (Fig. 3C), indicating that Gph mainly interacts with a specific form of GAPDH. These results provide evidence of a direct interaction between both proteins, and indicate that it depends on a given post-translational modification of GAPDH.

Since human GAPDH has been shown to be involved in repairing the DNA damage generated by bleomycin or alkylating agents (Azam et al., 2008), we examined whether Gph-GAPDH interacting complexes increased in cells challenged with bleomycin. To this end, induced exponential cultures of the *gph* mutant strain JA210 harbouring plasmid pGEX-Gph were treated with 8 μg/ml bleomycin in the presence of 50 μM $FeSO_4$, conditions that increase the number of DNA strand breaks to be repaired. At different times, aliquots of the culture were collected and processed to obtain the cell extracts. After

30 min exposure to bleomycin, the percentage of viable cells was found to be around 30%. Cell extracts were incubated with glutathione-sepharose 4B resin in PBS buffer. After extensive washing, GST-Gph was eluted with SDS-PAGE loading buffer. To evaluate GAPDH association, fractions were analysed by Western blotting with antibodies against GAPDH. For normalization, the same fractions were analysed in parallel with anti-GST antibodies (Fig. 5). In these experiments, the total amount of protein applied to the electrophoresis gel was lower than that used in the pull-down experiments presented in Fig. 3 in order to avoid saturation of the luminescent signal in the immunoblotting analysis. As shown in Fig. 5, the number of GAPDH molecules in complex with Gph increased in bleomycin-treated cells in a time-dependent manner. Overall, these results suggest that interaction between both GAPDH and Gph is triggered under activated DNA repair conditions. These proteins are probably partners of protein complexes involved in such processes.

Proteomic studies aimed at identifying protein interactions have great potential for suggesting new functions for a given protein. In fact, the presence of a protein in a multiprotein complex may be indicative that this protein has a second function (Jeffery, 2009). The study presented here may provide valuable clues to identify moonlighting functions for *E.coli* GAPDH. In this sense, the interaction with Gph points to a GAPDH role in cellular processes linked to DNA repair. In addition, interaction between these proteins may also suggest alternative GAPDH functions in quorum sensing signalling, since in enteric bacteria 2-phosphoglycolate is also generated as a degradation product of 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor of the autoinducer-2 (Taga, 2007; Xavier et al., 2007). In conclusion, this study may contribute to the identification of further moonlighting functions of *E. coli* GAPDH at the intracellular level.

Acknowledgments

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626

627 **Legends to figures**

628

629 Fig. 1. *In vivo* crosslinking of GAPDH-V5. TOP10 cells expressing GAPDH-V5 were
630 incubated with 1% formaldehyde for 20 min (FA +) or left untreated (FA -). TOP10 cells
631 without the recombinant plasmid pBAD-GapA were processed in parallel as control. (A)
632 Cell extracts were analyzed by Western blotting with anti-V5 or anti-GAPDH specific
633 antibodies. (B) Cell extracts obtained from TOP10 cells harbouring plasmid pBAD-GapA
634 after formaldehyde cross-linking were incubated at the indicated conditions to reverse
635 cross-links. Reversion was analyzed by Western blotting with anti-V5 antibodies.

636

637 Fig. 2. Immunoaffinity purification of GAPDH-V5 interacting proteins. (A) Purification of
638 GAPDH-V5 interacting proteins after formaldehyde cross-linking. TOP10 cells expressing
639 GAPDH-V5 were incubated with 1% formaldehyde for 20 min (+ FA) or left untreated (-
640 FA). GAPDH-V5 complexes were purified from the corresponding cell extracts under
641 astringent conditions using anti-V5 agarose beads. The purified complexes were incubated
642 at 95ºC for 30 min to reverse cross-links and then separated by SDS-PAGE. Proteins were
643 visualized by Sypro® Ruby staining. Twelve protein bands visible in the + FA lane
644 (labelled by white numbers) were excised from the gel and analyzed by MS. M, molecular
645 mass marker. (B) Two-dimensional analysis of proteins immunopreciptated with GAPDH-
646 V5 in the absence of formaldehyde cross-linking. Proteins were visualized by Sypro®
647 Ruby staining. Ten spots (labelled by white numbers) were excised from the gel and
648 analyzed by MS.

649

650

Fig. 3. Pull-down experiments showing binding of GAPDH to the ATP synthase α (AtpA) and β (AtpD) subunits. Cell extracts obtained from induced cells of strain AG1 bearing ASKA clone JW3710 (expressing His_6-AtpD) or ASKA clone JW3712 (expressing His_6-AtpA) were applied to a Ni^{2+}-NTA column. After extensive washing, elution of recombinant proteins was achieved with 300 mM imidazole (elution buffer). As a control, AG1 cell extract was applied to the Ni^{2+}-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 panel) or by Western blot with anti-GAPDH antibodies.

Fig. 4. Binding of GAPDH to Gph. (A) Pull-down experiments showing binding of GAPDH to Gph. Cell extracts (0.5 ml; 10 mg / ml) obtained from induced cells of strain BLB21(DE3) bearing plasmid pGEX (expressing GST) or plasmid pGEX-Gph (expressing GST-Gph) were passed over a glutathione-sepharose 4B column. After extensive washing, elution of GST or GST-Gph was achieved with glutathione elution buffer. Column fractions (FT: flow-through; W: wash; E: elution) were analyzed either by Coomassie blue staining (upper panel) or by Western blot with anti-GAPDH antibodies. (B) Co-purification of GAPDH with Gph. Gph was purified by affinity chromatography from induced cell extracts of strain BLB21(DE3) bearing plasmid pGEX-Gph (expressing GST-Gph). In this case, elution of Gph was achieved by factor Xa digestion. The eluted fractions were analyzed either by Coomassie blue staining (upper panel) or by Western blot with anti-GAPDH antibodies. The 35 kDa protein band was excised from the stained gel and identified as GAPDH by MS. (C) Far-Western analysis of binding of soluble Gph to PVDF-immobilized GAPDH. Samples containing 5.0 μg of purified GAPDH were

674 subjected to 2D gel electrophoresis, and the gel was either silver-stained (first panel) or
675 electroblotted. The PVDF membrane was reacted with purified Gph (5 μg/ml),
676 subsequently incubated with antibodies against anti-Gph and processed to visualize the
677 reactive bands (second panel). The third panel shows the result of this analysis when the
678 incubation step with Gph was omitted.
679
680 Fig. 5. Pull-down experiments to analyze interaction of GAPDH with Gph in cells
681 challenged with bleomycin. Induced cells of strain JA210 bearing plasmid pGEX-Gph
682 (expressing GST-Gph) were challenged with 8 μg/ml bleomycin-50 μM de $FeSO_4$. At the
683 indicated times, cell extracts were obtained. Samples were incubated with glutathione-
684 sepharose 4B resin. After extensive washing, elution of GST-Gph was achieved with
685 loading buffer 1x. Fractions were analyzed by Coomassie blue staining and by Western blot
686 with anti-GAPDH or anti-GST antibodies.

687 Table 1.
688 Proteins that copurified with GAPDH-V5 after *in vivo* formaldehyde cross-linking

Band	Protein	Accession	kDa	MASCOT score	Peptides sequenced	Function	Subcellular localization
1	D-ribose binding protein (RbsB)	P02925	28.5	236	5	D-ribose transport	Periplasm
1	Phosphoglycolate phosphatase	P32662	27.4	99	3	Metabolic enzyme	Cytoplasm
2	GAPDH	P0A9B2	36.1	447	11	Metabolic enzyme	Cytoplasm
2	OmpC	P06996	40.4	91	3	Porin	Outer membrane
3	GAPDH-V5	--	--	347	12	Recombinant protein	--
4	EF-Tu	P0CE47	43.3	503	14	Protein synthesis	Cytoplasm
5	Enolase	P0A6P9	45.6	238	5	Metabolic enzyme	Cytoplasm
6	Tryptophanase	P0A853	53.1	183	5	Metabolic enzyme	Cytoplasm
6	ATP synthase β subunit	P0ABB4	50.3	98	3	ATP synthesis	Inner membrane
7	Aldehyde dehydrogrenase (AldA)	P25553	52.4	284	6	Metabolic enzyme	Cytoplasm
7	Trigger factor	P0A850	49.0	115	4	Chaperone	Cytoplasm
7	Pyruvate kinase	P0AD61	51.6	97	3	Metabolic enzyme	Cytoplasm
8	Non identified	--	--	--	--	--	--
9	DnaK	P0A6Y8	69.1	510	10	Chaperone	Cytoplasm
10	Transketolase	P27302	72.4	105	5	Metabolic enzyme	Cytoplasm
11	Aconitase	P25516	94.0	135	3	Metabolic enzyme	Cytoplasm
12	Non identified	--	--	--	--	--	--

689
690

691 Table 2.
692 Proteins that copurified with GAPDH-V5 in the absence of formaldehyde cross-linking
693

Spot	Protein	Accesion	kDa	pI	Score	Peptides sequenced	Function	Subcellular localization
1	ATP synthase α subunit	P0ABB0	55.2	6.13	608.59	29	ATP synthesis	Membrane bound
1	Anthranilate synthase, component II	P00904	56.8	6.52	74.04	10	Trp biosynthesis	Cytoplasm
1	ATP-binding protein AraG	P0AAF3	55.0	6.38	59.09	10	L-Arabinose transport	Cytoplasm
1	ATP-binding protein RbsA	P04983	55.0	6.14	50.91	11	Function	Cytoplasm
1	Dihydrolipoyl dehydrogenase (lpdA)	P0A9P0	50.7	6.15	49.10	11	Pyruvate DH component	Cytoplasm
2	Non-identified	--	--	--	--	--	--	--
3	ATP synthase β subunit	P0ABB4	50.3	5.01	1150	40	ATP synthesis	Membrane bound
4	OmpF	P02931	39.3	4.96	156.9	19	Porin	Outer membrane
5	OmpC	P06996	40.3	4.82	445.05	31	Porin	Outer membrane
5	GAPDH	P0A9B2	35.5	7.11	153.96	19	Metabolic enzyme	Cytoplasm
6	GAPDH	P0A9B2	35.5	7.11	629.11	29	Metabolic enzyme	Cytoplasm
7 / 9 /10	GAPDH proteolysis fragments	P0A9B2	--	--	--	--	--	--
8	D-tagatose-1,6-bisphosphoaldolase, GatY	P0C8J6	30.8	6.34	259.55	21	Metabolic enzyme	Cytoplasm

694
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697

698

FIGURE 1

699

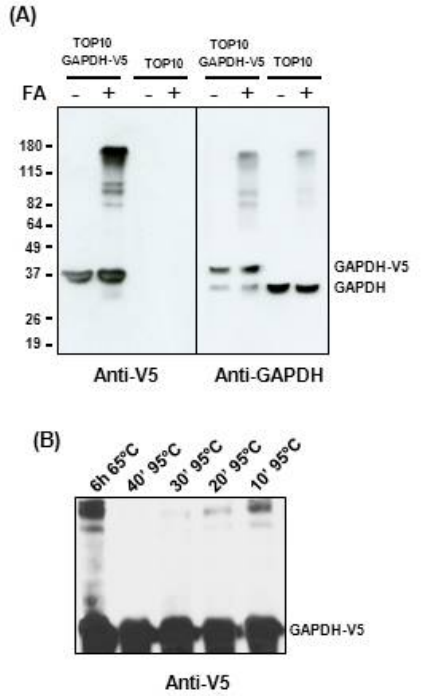
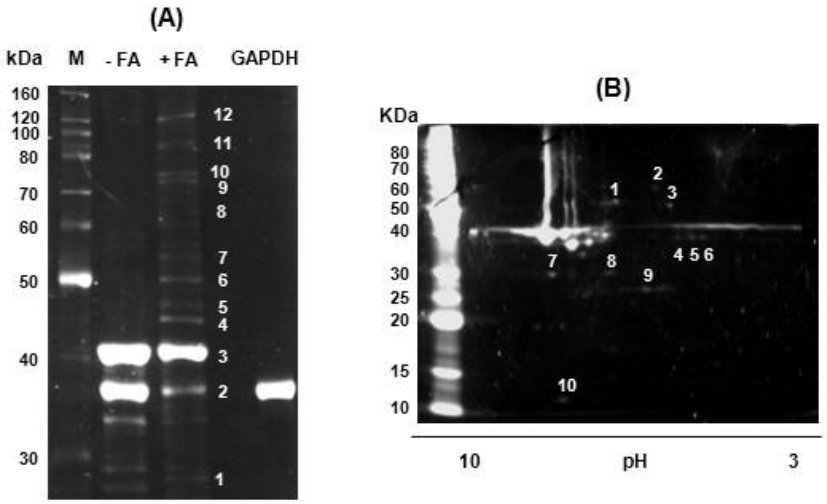


FIGURE 2


(A)
kDa
M
- FA
+ FA
GAPDH
160
120
100
80
70
60
50
40
30
12
11
10
9
8
7
6
5
4
3
2
1
(B)
KDa
80
70
60
50
40
30
25
20
15
10
1
2
3
4 5 6
7
8
9
10
10
pH
3

700

FIGURE 3

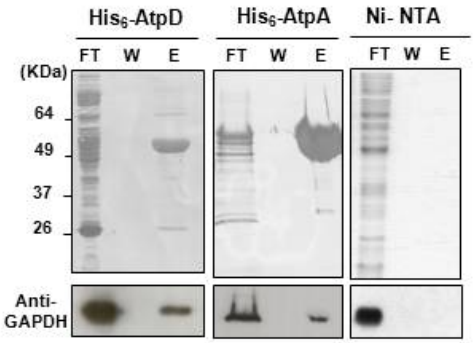
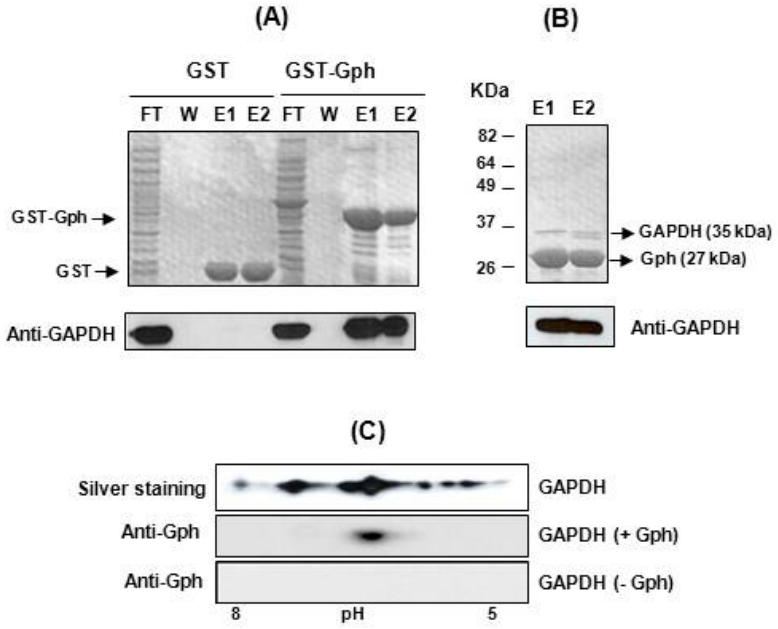
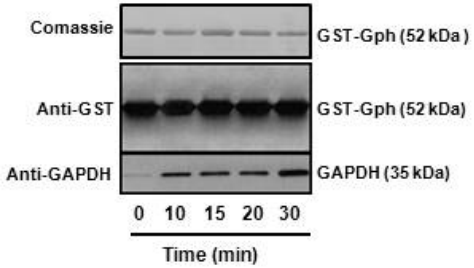

His6-AtpD
His6-AtpA
Ni- NTA
FT W E
FT W E
FT W E
(KDa)
64
49
37
26
Anti-
GAPDH

FIGURE 4


(A)
GST
GST-Gph
FT W E1 E2 FT W E1 E2
GST-Gph
GST
Anti-GAPDH
(B)
KDa
E1 E2
82
64
49
37
26
GAPDH (35 kDa)
Gph (27 kDa)
Anti-GAPDH
(C)
Silver staining
GAPDH
Anti-Gph
GAPDH (+ Gph)
Anti-Gph
GAPDH (- Gph)
8
pH
5

703

FIGURE 5


Comassie
GST-Gph (52 kDa)
Anti-GST
GST-Gph (52 kDa)
Anti-GAPDH
GAPDH (35 kDa)
0 10 15 20 30
Time (min)