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From famine to feast: the role of methylglyoxal production in *Escherichia coli*

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Summary

The enzyme methylglyoxal synthase (MGS) was partially purified from *Escherichia coli* extracts, and the amino-terminal sequence of candidate proteins was determined, based on the native protein being a tetramer of about 69 kDa. Database analysis identified an open reading frame in the *E. coli* genome, YccG, corresponding to a protein of 16.9 kDa. When amplified and expressed from a controlled promoter, it yielded extracts that contained high levels of MGS activity. MGS expressed from the *trc* promoter accumulated to approximately 20% of total cell protein, representing approximately 900-fold enhanced expression. This caused no detriment during growth on glucose, and the level of methylglyoxal (MG) in the medium rose to only 0.08 mM. High-level expression of MGS severely compromised growth on xylose, arabinose and glycerol. A mutant lacking MGS was constructed, and it grew normally on a range of carbon sources and on low-phosphate medium. However, the mutant failed to produce MG during growth on xylose in the presence of cAMP, and growth was inhibited.

Introduction

The role of methylglyoxal (MG) in cell physiology has been the subject of study for many years. Although known to be cytotoxic, it has been suggested to be a growth regulator (Együd and Szent-Györgyi, 1966) and to act as a bypass pathway for glycolysis (Hopper and Cooper, 1971). Methylglyoxal is extremely toxic to cells, and a balance of production and detoxification must be maintained. The toxicity is still poorly understood at the molecular level, but this electrophile has the capacity to modify DNA and proteins leading to inactivation of cells (Krymkiewicz *et al*., 1971; Lo *et al*., 1994; Papoulis *et al*., 1995). *Escherichia coli* cells, stimulated to produce MG by deregulation of the transport and metabolism of glucose-6-phosphate, arabinose, gluconate, glycerol and xylose, rapidly lose viability (Freedberg *et al*., 1971; Rekarte *et al*., 1973; Ackerman *et al*., 1974; Kadner *et al*., 1992). Growth inhibition is usually observed when MG passes a threshold concentration of 0.3 mM, and cell death occurs once the external concentration exceeds 0.6 mM (Ferguson *et al*., 1996). In *E. coli*, there are at least two levels of protection against MG: the activation of glutathione-gated K^+ channels (Ferguson *et al*., 1993) and the detoxification by reaction with glutathione followed by the action of glyoxalase I and II to produce D-lactate and free glutathione (Cooper, 1984). These two mechanisms are linked, as the product of glyoxalase I is the major activator of the KefB channel (MacLean *et al*., 1998). In addition to the glutathione-based detoxification, there are also glutathione-independent detoxification enzymes (Cooper, 1984; Misra *et al*., 1995; 1996), but these play only minor roles in the cell (MacLean *et al*., 1998).

The toxicity of MG suggests that the control over the activity of MGS is critical for cell viability and that it might be exploited as a new target for antibacterial therapy. It is a widespread enzyme, being found in all enteric bacteria tested (Hopper and Cooper, 1971), in *Pseudomonas saccharophila* (Cooper, 1974), *Pseudomonas doudoroffi* (Baumann and Baumann, 1975), in the Gram-positive organisms *Clostridium tetanomorphum* and *Clostridium pasteurianum* (Cooper, 1975), in a number of archaebacteria (Oren and Gurevich, 1995), in several yeast species (Babel and Hofmann, 1981) and in goat liver (Ray and Ray, 1981). Previous studies have established that MGS is a low-abundance protein in *E. coli* and that its activity is tightly regulated by homotropic activation by its substrate dihydroxyacetone phosphate (DHAP) and by a negative allosteric effector, phosphate (Hopper and Cooper, 1971). Other weaker inhibitors include 3-phosphoglycerate (PGA), phosphoenolpyruvate (PEP) and pyrophosphate. Regulation by phosphate can be equated with the possible role of MGS as a glycolytic bypass. Phosphate limitation of cells would be expected to lead to the accumulation of intermediates at the level of glyceraldehyde-3-phosphate, as the next enzyme in glycolysis has phosphate as a substrate. Routing carbon flux via MGS

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Table 1. Growth inhibition consequent upon overexpression of MGS.

Carbon source		Frag1/pTrc99A		Frag1/pST1	
		No IPTG	IPTG	No IPTG	IPTG
Glucose	Specific activity ($U\,mg^{-1}$ protein)	0.11 ± 0.03	0.11 ± 0.02	2.2 ± 0.16	104 ± 23
	Growth rate (h^{-1})	0.77 ± 0.03	0.76 ± 0.01	0.79 ± 0.02	0.72 ± 0.03
Glycerol	Specific activity ($U\,mg^{-1}$ protein)	0.12 ± 0.017	0.13 ± 0.02	3.0 ± 0.79	56 ± 14
	Growth rate (h^{-1})	0.51 ± 0.03	0.54 ± 0.05	0.52 ± 0.03	a
Xylose	Specific activity ($U\,mg^{-1}$ protein)	0.09 ± 0.005	0.11 ± 0.01	3.0 ± 0.5	66 ± 13
	Growth rate (h^{-1})	0.61 ± 0.02	0.63 ± 0.02	0.53 ± 0.02	a

Cultures were grown overnight in a shaking incubator and then diluted into fresh, prewarmed growth media, grown to mid-exponential phase ($OD_{650} = 0.4$), and 3 mM IPTG was added to induce the *trc* promoter. Cells were harvested 2 h after induction, and crude extract was prepared. **a.** Growth inhibition was progressive, not allowing a single valid growth rate to be deduced. The data are means plus SD from at least three experiments, except in the case of xylose, for which only two data sets were generated.

and the glyoxalases would enable cells to regenerate phosphate, while maintaining carbon flux through the cell. The enzyme has been purified from *E. coli* and was reported to be an oligomeric enzyme of about 69 kDa (Hopper and Cooper, 1972). However, the gene for the enzyme has not been described, and mutants lacking MGS have not been isolated.

In this study, the structural gene for MGS has been identified and a null mutant and a strain overexpressing MGS have been constructed. Analysis of their growth and survival has been undertaken to establish the role of the enzyme in cell physiology.

Results

Identification of the gene encoding methylglyoxal synthase

MGS was partially purified from *E. coli* extracts. Purification to homogeneity by previously published methods (Hopper and Cooper, 1971) was not accomplished, largely because of large and variable losses of activity in the initial stages of the purification. Rapid, partial purification by a combination of Phenyl Sepharose, Reactive Red and Superose 12 gel filtration chromatography was performed, and the N-terminal sequence of candidate proteins was determined. MGS has previously been reported to be a tetramer of approximately 69 kDa (Hopper and Cooper, 1971); therefore, proteins that had apparent molecular masses in the range of 17 kDa, the potential subunit size for the MGS monomer, were analysed. The combination of Phenyl Sepharose and Reactive Red chromatography achieved approximately 13-fold purification and yielded six candidate proteins. Subsequent separation by Superose 12 chromatography resulted in fractions that contained high levels of MGS activity (approximately $15\,U\,mg^{-1}$ protein compared with approximately $0.2\,U\,mg^{-1}$ protein in crude extract, a 75-fold enrichment) and in which two of the six candidates were prominent. One candidate was found to be a well-characterized gene product, FkpA (Horne and Young, 1995). The N-terminal sequence of the other, MELTTRTLPARKHIALVAHDHCKQ, was found to be 87% identical to the product of an unassigned open reading frame (ORF), *yccG*, adjacent to *helD* (Wood and Matson, 1989). The differences between the sequences lay entirely in the N-terminal three amino acids. This sequence was present in the −1 reading frame, and omission of a single base in the second codon after the ATG (from ATGAAACTG to ATGAACTG) would give the predicted protein sequence identified by N-terminal sequencing of our candidate protein. Subsequently, the *E. coli* genome sequencing project has completed this region and their DNA sequence (ECAE000198_) has the DNA sequence consistent with our protein sequence data. The corrected DNA sequence and projected protein sequence have been submitted to EMBL (accession number Y11249).

The correct *yccG* ORF was amplified by polymerase chain reaction (PCR) and cloned under the *trc* promoter in plasmid pTrc99A to create plasmid pST1. Induction of expression of *yccG* by growth in LB in the presence of 3 mM IPTG produced cell extracts that contained 575-fold greater MGS activity than parent cells, increasing from $0.11 \pm 0.01\,U\,mg^{-1}$ protein to $63.2 \pm 0.8\,U\,mg^{-1}$ protein. A unique prominent protein of 17 kDa was observed by SDS–PAGE in extracts derived from induced cells grown in defined medium. MGS activity in these extracts was increased 600- to 900-fold (Table 1; Fig. 1; data not shown). These data confirm the identity of the *yccG* ORF as encoding methylglyoxal synthase, and we propose that the *yccG* ORF be renamed *mgsA* (accession number Y11249).

We sought to determine the physiological role of MGS through overexpression of the enzyme and through creation of an *mgsA* null mutant.

Physiological effects of overproduction of MGS

Methylglyoxal is extremely toxic to cells (Együd and Szent-Györgyi, 1966). The enzyme is normally expressed at relatively low levels in cells and is subject to allosteric regulation, namely inhibition by phosphate and homotropic

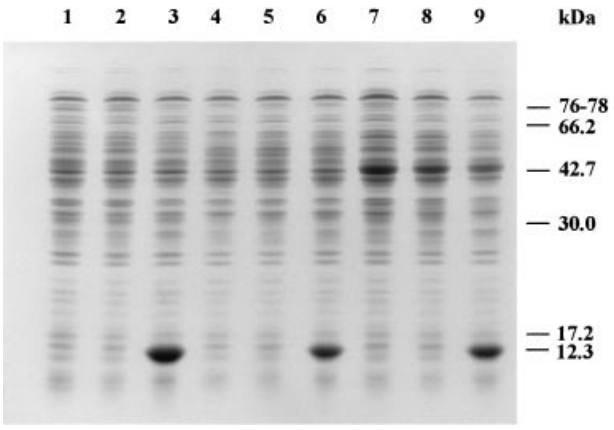

Fig. 1. Production of MGS in *E. coli* cells. Cells were grown to mid-exponential phase in the presence or absence of IPTG, and samples were harvested and suspended in gel loading buffer. Samples were incubated at 100°C for 5 min before separation of the proteins on a 10% SDS–PAGE gel, followed by staining with Coomassie blue. Lanes 1–3, glucose-grown cells; lanes 4–6, glycerol-grown cells; lanes 7–9, xylose-grown cells; lanes 1, 4 and 7, Frag1/pTrc99A, induced with 3 mM IPTG for 2 h; lanes 2, 5 and 8, Frag1/pST1; lanes 3, 6 and 9, Frag1/pST1 induced with 3 mM IPTG for 2 h.

activation by its substrate, dihydroxyacetone phosphate (Hopper and Cooper, 1972). Thus, overexpression might have a deleterious effect under conditions in which phosphate is depleted and/or DHAP levels are high. In complex medium, the growth rates of cultures overexpressing MGS were identical to those of the parent (data not shown). A preliminary screen of growth on different carbon sources on solid media suggested that low-level overexpression of MGS (approximately 20-fold) slightly impaired growth on xylose and glycerol, but not on glucose, maltose, arabinose, sorbitol, ribose, glucuronate, lactate and succinate. However, when the overexpression was coupled with the presence of cAMP (see *Experimental procedures*), growth inhibition was evident on arabinose, glycerol and xylose, and weak inhibition was seen with ribose. The physiological effects of MGS overproduction were investigated in more detail by monitoring growth, viability, MGS activity and MG production during growth on minimal medium (K_{10}) with different carbon sources and in medium limited for phosphate.

Growth on glucose. Induction of *mgsA* expression by growth with IPTG led to a rise in the enzyme activity (930-fold compared with the control strain) in glucose-grown cultures (Table 1). MGS overexpression in K_{10} glucose medium was found to have no obvious effect on growth. No MG accumulation was observed in non-induced cells. In induced cells, the MG concentration only rose to approximately 0.08 mM at the end of the exponential phase and thereafter declined (data not shown). These data indicate that the actual accumulation of MGS, which constitutes approximately 20% of total cell protein in the induced culture (Fig. 1), does not significantly affect the growth rate.

Growth on glycerol. Growth in liquid medium with glycerol as sole carbon source was largely unaffected by a 25-fold increase in MGS activity (Table 1). In the presence of IPTG, MG accumulated in the medium, and growth was progressively inhibited (Fig. 2A). Growth on glycerol ceased when the concentration of MG in the medium reached 0.3 mM (Fig. 2A and B), and cultures lost viability when the external concentration reached 0.6 mM (Fig. 2B and C). When growth ceased, MG production accelerated (Fig. 2A and B).

Growth on xylose. Growth inhibition (approximately 12% reduction in specific growth rate; Table 1) was observed even with a moderate increase (33-fold) in the expression of MGS. Induction of MGS expression with IPTG led to progressive growth inhibition (Fig. 3A). However, this did not correlate with the MG concentration (Fig. 3B), which remained relatively low throughout the experiment; after 200 min, the MG concentration was only 0.2 mM. Thus, unlike the glycerol cultures, those grown on xylose did not produce high levels of MG but were profoundly inhibited (Fig. 3A and B; compare with Fig. 2A and B; note that identical scales have been used in Figs 2 and 3 to facilitate comparison). Viability remained high throughout the experiment (Fig. 3C), and this correlates with the low level of MG accumulated in the medium. Thus, the growth inhibition seen in the xylose-grown culture appears to have a different basis to that found for glycerol-grown cultures. It is notable that a prominent protein band (approximately 50 kDa) was induced during growth on xylose and exhibited a much reduced accumulation after the induction of MGS expression (Fig. 1, lanes 7–9). The growth of *E. coli* on xylose is strongly dependent on the activity of xylose isomerase and xylulose kinase (Lawlis *et al*., 1984), which have molecular masses of approximately 50 kDa, and on transketolase (Draths *et al*., 1992). Reduced expression of any of these proteins could account for the lower growth rate in cells expressing high levels of MGS.

Phosphate limitation. Phosphate limitation might be expected to lead to MG production as a result of loss of inhibition of MGS by phosphate. However, MG accumulation was slow under conditions of phosphate limitation in the parent. The rate of MG accumulation was significantly faster when MGS was overexpressed, but the rate was not sustained beyond 90 min starvation and, thereafter, there was a slow decline in the level of MG in the medium (Fig. 4). There was no loss of viability provoked by the

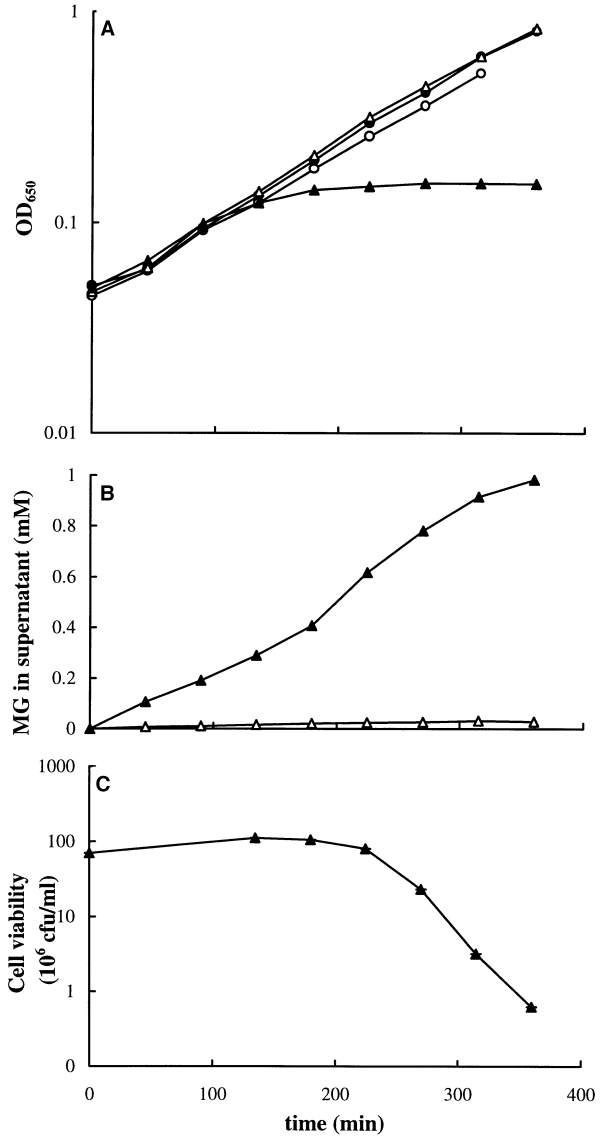

Fig. 2. Overexpression of MG synthase in minimal medium with glycerol as carbon source: effect on growth, MG accumulation in the medium and cell viability. Cultures were grown to log phase in K_{10} glycerol and then diluted into fresh prewarmed medium in the absence (open symbols) and in the presence of 3 mM IPTG (closed symbols). Circles, Frag1/pTrc99A; triangles, Frag1/pST1.
A. Cell growth measured as optical density (OD_{650}).
B. MG production.
C. Cell viability.

higher level of MG accumulation in strains overexpressing MGS under conditions of phosphate limitation (data not shown).

Creation of an MGS null mutant

An *mgsA* null mutant was created by inserting a kanamycin cassette into the *Bam*HI site approximately 270 bp from the 5′ end of the *mgsA* gene, followed by integration of the disrupted gene into the chromosome of *E. coli* strain JC7623 (see *Experimental procedures*). The resulting *mgsA*::*kan* insertion was transduced into strain Frag1 to create an isogenic MGS null mutant strain, MJF397 (Frag1, *mgs*A::*kan*). MGS activity was not detectable in extracts, consistent with the inactivation of the structural gene for MGS. Elimination of MGS activity did not compromise the growth or survival of cells on phosphate-limited medium (data not shown). On solid media, no growth phenotype was observed, irrespective of the carbon source.

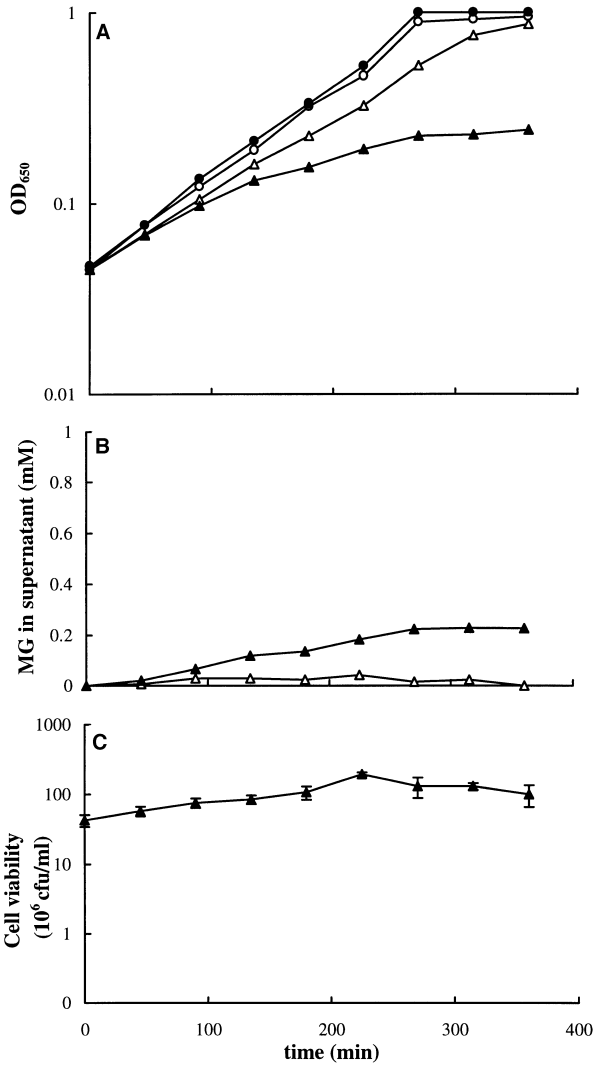

Fig. 3. Overexpression of MG synthase in minimal medium with xylose as carbon source: effect on growth, MG accumulation in the medium and cell viability. Cultures were grown to log phase in K_{10} xylose and then diluted into fresh prewarmed medium in the absence (open symbols) and in the presence of 3 mM IPTG (closed symbols). Circles, Frag1/pTrc99A; triangles, Frag1/pST1.
A. Cell growth was measured as optical density (OD_{650}).
B. MG production.
C. Cell viability.

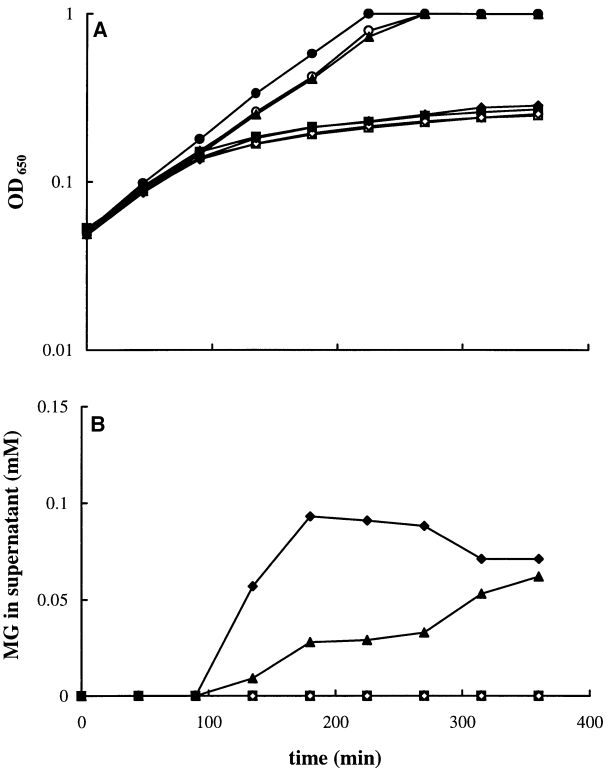

Fig. 4. Growth and survival of Frag1 and MJF397 (MGS^-) under phosphate-limiting conditions. Cultures were grown overnight in glucose-containing HEPES medium containing 660 μM phosphate and then washed and diluted into HEPES medium containing either excess (660 μM) phosphate (open symbols) or limiting (40 μM) (closed symbols) phosphate. Circles, Frag1; triangles, MJF397 (MGS^-).
A. Cell growth was measured as optical density (OD_{650}).
B. Cell viability.

When a filter disk carrying cAMP was placed in the centre of the plate (see *Experimental procedures*), a transient zone of growth inhibition was observed on xylose minimal medium but not with other carbon sources. Similarly, in liquid medium, a growth phenotype was only observed when cells were grown with xylose as sole carbon source in the presence of cAMP (Fig. 5). In Frag1 cells, this causes overexpression of the enzymes of xylose transport and metabolism, leading to methylglyoxal synthesis and excretion (Ackerman *et al*., 1974; Ferguson *et al*., 1996). Addition of cAMP to *E. coli* cells growing on xylose led to progressive growth inhibition followed by cell death once the MG concentration rose above approximately 0.6 mM (Fig. 5). In contrast, growth of the MGS^- mutant strain was inhibited within 20 min of the addition of cAMP (Fig. 5). There was no synthesis of MG in the mutant and no loss of viability (Fig. 5). There was no significant increase in viable cell numbers, although the OD_{650} continued to increase slowly. Thus, it appears that MGS is required under conditions that increase the rate of xylose metabolism.

Discussion

There is an inherent paradox in the possession by bacteria of an enzyme, methylglyoxal synthase, that produces an extremely toxic product. It has been rationalized that methylglyoxal production might be required for growth (Együd and Szent-Györgyi, 1966). However, under most conditions, the growth and survival of the *mgsA* mutant was quite normal. From our study, the primary role of MGS in *E. coli* appears to be the limitation of the accumulation of phosphorylated sugars. The production of MG

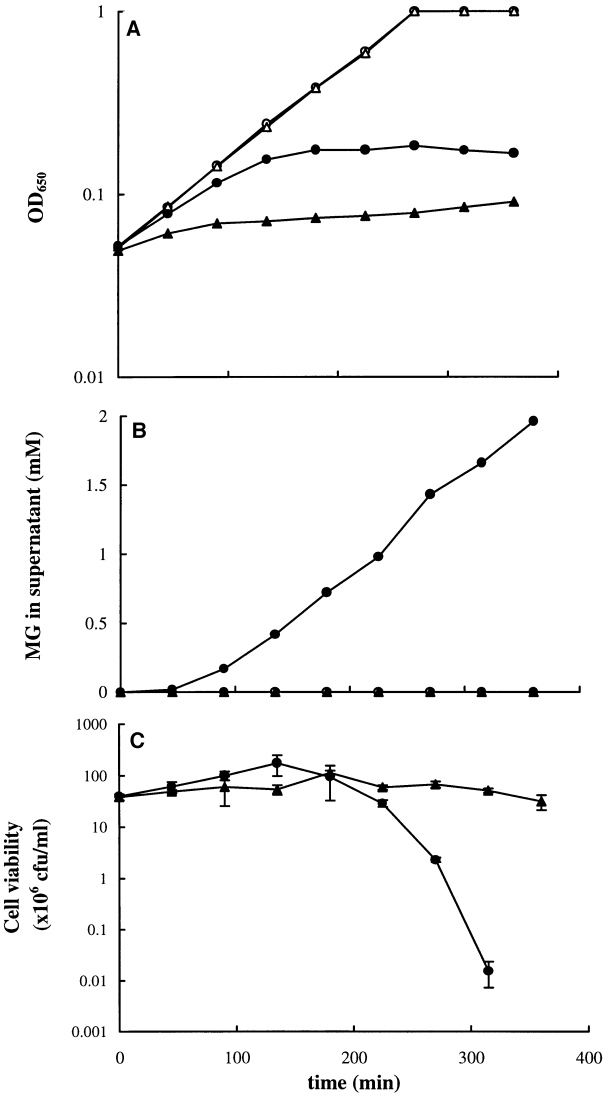

Fig. 5. Growth and survival and MG production of Frag1 and MJF397 during elevated xylose metabolism. Cultures were grown to log phase in K_{10} xylose and then diluted into fresh prewarmed medium in the presence (closed symbols) and in the absence (open symbols) of 2 mM cAMP. Circles, Frag1; triangles, MJF397.
A. Cell growth was measured as optical density (OD) at 650 nm.
B. MG production.
C. Cell viability.

has most frequently been found when regulation of carbon source utilization has been disrupted either by mutations or by manipulation of the cAMP concentration in the environment (Zwaig *et al*., 1970; Freedberg *et al*., 1971; Rekarte *et al*., 1973; Ackerman *et al*., 1974; Melton *et al*., 1981; Kadner *et al*., 1992). Such conditions lead to release from self-repression, consequent higher activities of inducible enzymes causing increased flux through the upper segment of glycolysis (Katz and Englesberg, 1971; Ackerman *et al*., 1974; Cooper, 1974). Kadner *et al*. (1992) used non-metabolizable sugar phosphates to differentiate between the toxicity of MG production and inhibition of growth through the accumulation of phosphorylated sugars. Deregulated entry of glucose 6-phosphate kills *E. coli* cells, primarily because of MG production (Kadner *et al*., 1992). However, accumulation of non-metabolizable sugar phosphates also causes growth inhibition in mutants of *E. coli* without loss of viability (Kadner *et al*., 1992). This is similar to the pattern seen with xylose and cAMP in our study with the parent (inhibition and death associated with MG production) and the *mgsA* null mutant (inhibition but no loss of viability). It is known that the addition of cAMP to *E. coli* cells growing on xylose causes a 40% increase in the expression of xylulolosekinase and xylose isomerase (Ackerman *et al*., 1974). The increased flux from xylose to ribulose-5-phosphate may reduce the phosphate pools and increase the DHAP pools sufficiently to activate MGS. Triose phosphate isomerase favours the formation of DHAP (Gottschalk, 1985); any substrate that leads to high pools of either of these triose phosphates could favour MG production. Thus, in the *mgsA* null mutant, growth inhibition would be caused by the accumulation of sugar phosphates. MGS activity would be required simply to recycle phosphate and would diminish the build-up of sugar phosphates.

In our study, we saw only very limited accumulation of MG under conditions of imposed phosphate limitation. These data can be accounted for in the above model for the role of MGS in cells by the fact that some MG production may be masked by the very efficient glyoxalase systems (Cooper, 1974; Misra *et al*., 1995; MacLean *et al*., 1998) and by limitation for DHAP. Lack of phosphate will limit the flux through glyceraldehyde-3-phosphate (PGAL) dehydrogenase, which will reduce the availability of PEP for the entry of phosphotransferase substrates and of ATP for the phosphorylation of all substrates. Thus, the substrate for MGS should not be maintained at high levels under conditions of phosphate limitation.

All the conditions that lead to excessive production of MG are associated with loss of control over carbon entry. Such an event is most likely in the natural state when an organism previously adapted to an environment containing only low levels of carbon sources (e.g. sugars) suddenly encounters high levels. Xylose, although an abundant carbon source in natural environments, is usually in polymeric form and is made available by enzymatic digestion. Transitions from low abundance to high are possible, and indeed likely, given that there are few controls exerted over secreted enzymes. Further, *E. coli* cells preadapted to a carbon source exhibit co-metabolism when simultaneously fed that carbon source and glucose, leading to elevation of both hexose phosphate and DHAP pools compared with cells growing on glucose alone (Lowry *et al*., 1971). Lack of MGS might impede the adaptation from, say, xylose or glycerol to glucose. Thus, the possession of MGS can be seen as an essential adjunct to the selective utilization of carbon sources and the control of gene expression by monitoring of the rate of sugar flux through the cell.

It is clear from our studies that a relatively low abundance of MGS in *E. coli* cells may itself be protective, as overexpression of the enzyme can be toxic during growth on carbon sources subject to self-repression. The maximum activity of MGS determined in our study was 0.1–0.2 U mg^{-1} cell protein, which is similar to the lowest activity for enzymes of the glycolytic sequence (range 0.1–4 U mg^{-1} protein; Gottschalk, 1985). The DHAP pools in *E. coli* have been estimated to be approximately 0.5 mM on glucose-defined medium and as high as 2 mM on glycerol (Lowry *et al*., 1971). The K_m of MGS for DHAP is in this range and, thus, a high abundance of this enzyme could pose a significant threat to the organism. By possessing a low abundance of MGS, the rate of synthesis of MG can be maintained at a relatively non-toxic level, while still reducing the stress associated with sugar phosphate accumulation. This is augmented by the negative feedback provided by inhibition by phosphate and potentially also by PGA and PEP. Under extremely enhanced carbon source metabolism, the production of MG results in a temporary relief from the stress caused by phosphorylated intermediates. This allows the cells to grow until dilution of the inducible metabolic enzymes and/or entry into an environment less abundant in free carbon relieves the stress. In the laboratory, conditions are poised such that the accumulation of MG results in growth inhibition and eventually cell death. This implies that MGS is important for coping with rapid changes in the environment between 'famine' and 'feast' conditions.

We conclude that MGS is not an essential enzyme under conditions of glucose metabolism. However, it does have a major function in reducing the potential imbalance in metabolism at the junction between the inducible and constitutive elements of the glycolytic sequence. This proposal is consistent with the widespread occurrence of MGS in bacteria (Hopper and Cooper, 1971) and the occurrence of similar gene products in a range of Gram-positive and Gram-negative organisms. Database searches with the *E. coli mgs*A gene revealed homologues in *Haemophilus*

influenzae, D641169 (58.6% similarity), *Bacillus subtilis*, P42980 (42.3% similarity), *Brucella abortus*, BAU21919_2 (44.8% similarity) and *Synechocystis*, SYCSLLLH_17 (37.9% similarity). The sequence similarity is extensive along the length of the protein. Interestingly, there is no homologue of MgsA in the yeast genome, and it is known that the regulation of this activity in yeast is quite different from that in *E. coli* (Murata *et al*., 1985). Thus, MGS may be an excellent target for antibacterial agents.

Experimental procedures

Bacterial strains and growth conditions

E. coli Frag1 (F^-, *gal*, *lacZ*, *rha*, *thi*) was used as the wild-type strain. MJF397 (Frag1, *mgsA*::kan) was constructed as described below. JC7623 (*recB21*, *recC22*, *sbcB15*; Kushner *et al*., 1971) was used for the recombination of linearized DNA into the chromosome.

Medium K_x (Epstein and Kim, 1971), where × is the concentration of potassium (mmol l^{-1}) or HEPES medium (Tommassen and Lutgenberg, 1980), containing 660 μM or 40 μM phosphate was used for growth experiments as indicated. The carbon sources were glucose (0.2%, w/v), glycerol (0.4%, w/v), arabinose (0.2%) or xylose (0.2%, w/v), and all experiments were carried out at 37°C. Cultures were grown overnight in a shaking incubator and then diluted into fresh growth medium, grown to mid-exponential phase (OD_{650} = 0.4; measured using a Pharmacia-LKB Novospec II) and diluted to OD_{650} = 0.04 with fresh prewarmed growth medium. Additions of IPTG were made as indicated. For the analysis of the effect of limited phosphate on MJF397, cells were grown overnight in HEPES medium containing 660 μM phosphate and then diluted into fresh HEPES containing either 40 μM (phosphate-limited culture) or 660 μM phosphate (excess), resulting in final phosphate concentrations of approximately 62 μM and 660 μM respectively.

Cultures carrying plasmids were always grown in the presence of ampicillin (25 μg ml^{-1}). MJF397 was grown overnight in the presence of kanamycin (50 μg ml^{-1}). Measurements of growth were made by monitoring the OD_{650} of the cell suspension. Aliquots to determine cell viability and MG accumulation in the medium were taken at intervals.

Viable counts

Cell viability was determined by making serial dilutions into K_{10} medium or HEPES medium lacking any carbon source and spotting 5 μl aliquots (triplicate) on K_{10} plates containing the appropriate carbon source (Ferguson *et al*., 1993). Incubations were for 24–48 h at 37°C depending on the carbon source. The mean and standard deviation of the number of viable colonies ml^{-1} is plotted on all figures. The limit of detection of this method is 200 viable cells ml^{-1}. All experiments were repeated several times, but each figure represents a typical experiment.

Carbon source utilization patterns

Different carbon sources (final concentration 0.2% w/v, except for glycerol, which was 0.4% w/v) were added to defined medium (K_{10}) agar plates, and colonies were streaked from the perimeter to the centre of the plate. A filter disk (5 mm diameter) was placed in the centre of the plate, and either 5 μl of sterile H_2O or 5 μl of 2 M cAMP were placed on the disk. The plates were incubated for 24 h and the growth recorded.

Purification of MGS

The enzyme was purified from glycerol-grown cells of *E. coli* Frag1. The buffer used throughout the purification was 25 mM Tris, 25 mM *bis*-Tris propane, 1 mM EDTA, 1 mM KH_2PO_4, pH 7.5 (buffer A). At each stage, enzyme activity was determined and active fractions were pooled and concentrated where necessary, using either an Amicon cell with a PM 10 membrane or a Centricon-10 concentrator. Overnight cultures were harvested, washed with buffer and suspended in buffer (1 g ml^{-1} wet weight). The cells were disrupted by passage through a French press (three passes at approximately 15 drops min^{-1} at a cell pressure of 20 000 psi). The resulting extract was centrifuged at 30 000 × *g* for 30 min at 4°C. Extract was stored at −20°C until further purification. All further purification steps were performed at room temperature.

Hydrophobic interaction chromatography with Phenyl Sepharose. Crude extract (100 mg) was diluted 1:2 in buffer A containing 1 M NaCl and filtered (Millipore; 0.2 μm pore size). This suspension was than applied to a column (2.5 cm × 20.4 cm) of Phenyl Sepharose (Pharmacia Biotech) previously equilibrated with buffer A containing 1 M NaCl. The column was washed with 700 ml of buffer A containing 1 M NaCl, 420 ml buffer A and finally eluted with 150 ml of 30% (v/v) isopropanol in buffer A.

Affinity chromatography with Reactive Red. The concentrated pool from the Phenyl Sepharose column was applied to a column (2.5 cm × 10.2 cm) of Reactive Red 120 (Sigma) previously equilibrated with buffer A. The enzyme was eluted in the unbound fraction.

Gel filtration on Superose 12. Concentrated protein (0.5 mg in 200 μl) was applied to a Superose 12 HR 10/30 column (Pharmacia Biotech) previously equilibrated with buffer A containing 50 mM NaCl. The column was eluted at a flow rate of 0.5 ml min^{-1}.

Gel electrophoresis

Reducing sample buffer [40% (v/v) glycerol, 8% (w/v) SDS, 0.25 M Tris (pH 6.8), 0.1% (w/v) bromophenol blue, 0.75 M 2-mercaptoethanol] was mixed 1:1 with extract and boiled for 5 min. A 20 μl volume of this solution, containing approximately 25 μg of protein, was then loaded onto a 13% polyacrylamide gel. Gels were stained by incubation in Coomassie blue staining solution (PhastGel Blue R Tablets, Pharmacia Biotech) for 20 min, rinsed with distilled water and then destained with 10% ethanol/10% acetic acid mix for 2 h with several changes of the destain solution. The protein gels were scanned using an Epson GT-6500 flat bed black and white scanner, and the band intensities were determined using Phoretix 1D gel analysis software.

Protein sequencing

The sequencing of the N-terminal amino acid sequence of different protein bands was performed as described previously (Dunbar and Wilson, 1994) using blotting onto PVDF membrane (ProBlott; Applied Biosystems) and staining with amido black. Protein bands were cut out and analysed on an Applied Biosystems automated sequencer (model 477A).

DNA manipulation

Plasmid preparations were carried out using the QIAprep Spin Miniprep Kit (Qiagen) as described by the supplier. Restriction digestion, DNA ligation and transformation were carried out by standard protocols as described by Maniatis *et al*. (1982). DNA sequencing reactions were performed as detailed in the instructions supplied with the PRISM ready reaction dideoxy terminator cycle sequencing kit (Applied Biosystems or ABI). The cycle sequencing reactions were analysed on an Applied Biosystems 373A DNA sequencer. Results obtained from sequencing reactions were analysed using Lasergene (DNASTAR). Sequences were obtained at least three times from both strands.

Construction of MGS expression vector

The expression of *yccG* was obtained by cloning the gene in frame between the *Nco*I and *Sma*I sites of the expression vector pTrc99A (Pharmacia Biotech). The *yccG* ORF was amplified with primer MGS5 (TGTACACCATGGAACTGACGACTC), which was designed to contain an *Nco*I site (underlined) and the corrected sequence, and MGS4 (GGCGGAGCATGCCATCATCGTTGGCTTG). The 750 bp PCR product was than treated with Klenow to ensure that the 3′ ends were filled, and the DNA was purified. The PCR product was digested with *Nco*I and ligated into pTrc99A, previously digested with *Nco*I and *Sma*I. The ligation mixture was used to transform strain JM109, and colonies were screened by PCR using the vector-specific primer (TTGACAATTAATCATCCGGC) and MGS4. Plasmids with the expected insert were isolated, and the insert was sequenced using PCR-based cycle sequencing with vector-specific primer and primer MGS4. Each strand was sequenced at least four times.

Construction of mgsA null mutant

A 2 kb fragment containing *yccG* and the flanking regions on either side was amplified with primers MGS1250F (AGGCGAGCATGCTTCTGGAGAAAGG) and MGS3300R (AAACCGGAATTCTGACCACCTTGATTG), which contain the restriction sites *Sph*I and *Eco*RI respectively. The 2 kb PCR product was than treated with Klenow to ensure that the 3′ ends were filled, and the DNA was purified. The PCR product was then digested with these restriction enzymes and ligated with pUC18 (Pharmacia Biotech), previously digested with the same enzymes, to form plasmid pST2. The construct was digested with *Bam*HI, which has a single restriction site in the middle of *yccG* on pST2. A kanamycin cassette obtained from Pharmacia was also digested with *Bam*HI and inserted into pST2 to interrupt *yccG* (pST3). To allow recombination into the chromosome, pST3 was digested with *Sph*I, *Eco*RI and *Sca*I, which resulted in the linearization of the *yccG*::kan fragment, including a 1 kb flanking region on either side of the kanamycin cassette, and the disruption of the ampicillin resistance gene of the plasmid. This digest was then transformed into JC7623. Kanamycin-resistant, ampicillin-sensitive transformants were isolated. An additional screen using PCR with a kanamycin cassette-specific primer Kan92 (GATTCAGGCCTGGTATG) or Kan1121 (CCCGTTGAATATGGCTC) and MGS480F (GTCAGGGCATGCTGAAAGATATTGAAAG), which binds to the chromosome but not to the plasmid pST3, was performed. The kanamycin cassette insertion into *yccG* resulted in the complete loss of MGS activity in crude extract. The *yccG*::kan insertion was then transduced into Frag1 using phage P1 generating the strain MJF397. Enzyme assays in crude extract confirmed that MGS activity was abolished completely.

Crude extract after overproduction and knock-out of MGS

Cells were harvested and, after washing with buffer A, the pellet was suspended 100- to 200-fold concentrated. Glass beads (250 μl, 0.4 mm; BDH) were added to 300 μl of concentrated cell suspension and vortex mixed six times for 1 min with incubation on ice for 1 min between each mix. The resulting suspension was centrifuged in Eppendorf tubes for 15 min at 14 000 × *g* at 4°C. The supernatant was stored at −20°C.

MG assay

To measure the MG accumulation in the medium, 500 μl samples were taken at intervals, and the cells were removed by centrifugation in a microcentrifuge (14 000 × *g* for 1 min). The supernatant was transferred to a fresh Eppendorf tube and stored at −20°C until the assay was conducted. Methylglyoxal was assayed colorimetrically using 2,4-dinitrophenolhydrazine (2,4-DNPH) adapted from the MGS assay described below. Standards (10 μl containing 0–10 nmol of MG) and sample were added to microtitre plate wells, and 70 μl of distilled water plus 30 μl of 0.1% 2,4-DNPH in 2 M HCl was added. After 15 min incubation at room temperature, 140 μl of 10% NaOH was added. After incubation for a further 15 min at room temperature, absorbance was measured at 540 nm. Standards were assayed in duplicate and samples in triplicate.

MGS assay

MGS activity was measured spectrophotometrically using a linked assay in which the product MG was conjugated to glutathione and converted to S-lactoylglutathione by excess glyoxalase I as described previously (Racker, 1957; Hopper and Cooper, 1971). For the screening of fractions containing MGS during purification, a microtitre assay was developed with a reaction volume of 80 μl; this was adapted from a colorimetric assay with 2,4-DNPH (Wells, 1966; Cooper and Anderson, 1970). Standard solutions contained 0.5–10 nmol of MG in 40 mM imidazole buffer, pH 7.0. For the enzyme reaction, 20 μl of diluted extract (at least 10^{-1} diluted in 40 mM imidazole buffer, pH 7.0, to dilute out phosphate) was added into microtitre wells. The reaction was started with the addition

of 60 μl of 0.5 mM DHAP in 40 mM imidazole buffer, pH 7.0, at 20°C. After 10 min, 30 μl of 0.1% 2,4-DNPH in 2 M HCl was added to standards and samples. After 15 min incubation, 140 μl of 10% NaOH was added. After incubation for a further 15 min, absorbance was measured at 540 nm. Standards and samples were assayed in duplicate.

Materials

Fine biochemicals were purchased from Sigma, and components of defined media were from BDH. Restriction enzymes and *Taq* polymerase were purchased from Boehringer and Promega. DNA primers were synthesized by GENOSYS.

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