The critical role of S-lactoylglutathione formation during methylglyoxal detoxification in Escherichia coli

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Summary

Survival of exposure to methylglyoxal (MG) in Gram-negative pathogens is largely dependent upon the operation of the glutathione-dependent glyoxalase system, consisting of two enzymes, GlxI (gloA) and GlxII (gloB). In addition, the activation of the KefGB potassium efflux system is maintained closed by glutathione (GSH) and is activated by S-lactoylGSH (SLG), the intermediate formed by GlxI and destroyed by GlxII. Escherichia coli mutants lacking GlxI are known to be extremely sensitive to MG. In this study we demonstrate that a ΔgloB mutant is as tolerant of MG as the parent, despite having the same degree of inhibition of MG detoxification as a ΔgloA strain. Increased expression of GlxII from a multicopy plasmid sensitizes E. coli to MG. Measurement of SLG pools, KefGB activity and cytoplasmic pH shows these parameters to be linked and to be very sensitive to changes in the activity of GlxI and GlxII. The SLG pool determines the activity of KefGB and the degree of acidification of the cytoplasm, which is a major determinant of the sensitivity to electrophiles. The data are discussed in terms of how cell fate is determined by the relative abundance of the enzymes and KefGB.

Introduction

Bacteria have evolved elaborate and complex stress management strategies to minimize damage and thus, to enhance their survival during environmental changes (Booth, 2002). In addition, metabolic activity in itself can create significant stress, for example the production of hydrogen peroxide and oxygen radicals is a consequence of aerobic growth and the resulting oxidative damage requires both intrinsic and adaptive enzyme activities (Imlay, 2008; Korshunov and Imlay, 2010). Similarly, bacteria encounter electrophiles both as a metabolic consequence and as an environmental challenge. Among the most frequently encountered electrophiles is methylglyoxal (MG), which is produced by bacteria from sugars and amino acids and is believed to have a role in macrophage-mediated killing (Eskra et al., 2001; Eriksson et al., 2003; Ficht, 2003). MG is synthesized either from sugars by methylglyoxal synthase (MGS) (Totemeyer et al., 1998) or from threonine, serine and glycine by monoamine oxidase (Green and Lewis, 1968; Kim et al., 2004). In Escherichia coli the dominant route appears to be from sugars and arises when there is an accumulation of phosphorylated glycolytic intermediates above the level of 1,3-diphosphoglycerate and a lowering of the pool of inorganic phosphate (Hopper and Cooper, 1971; Ferguson et al., 1998; Totemeyer et al., 1998). MGS activity is determined by the balance between inorganic phosphate, which is a strong inhibitor, and dihydroxyacetone phosphate (DHAP), the substrate, which exhibits strong homotropic activation (Hopper and Cooper, 1971). Thus, production of MG only occurs when there is simultaneous depletion of phosphate and extremely high concentrations of DHAP, conditions that arise when sugar metabolism is strongly stimulated leading to excess carbon flow into the upper end of glycolysis (Freedberg et al., 1971; Ackerman et al., 1974; Burke and Tempest, 1990; Kadner et al., 1992; Russell, 1993). For E. coli, accumulation of MG above ~0.3 mM in the medium results in growth inhibition and at levels above ~0.6 mM the survival of cells is affected. Damage to DNA and to proteins has been observed (Krymkiewicz, 1973; Colanduoni and Villafranca, 1985; Ferguson et al., 2000) and both may contribute to cell death.

Protection against electrophiles is multifactorial with contributions from glutathione (GSH), detoxification enzymes, DNA repair enzymes, peptide export systems and regulated K+ efflux systems (Ferguson and Booth, 1998; MacLean et al., 1998; Ko et al., 2005; Xu et al., 2006; Sukdeo and Honek, 2008). In E. coli, detoxification is primarily effected by the GSH-dependent glyoxalase system (GlxI and GlxII; products of the unlinked gloA and gloB genes) and their integration with the GSH adduct-gated KefGB K+ export systems (Fig. 1). Other enzymatic

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systems, particularly a range of oxidoreductases (Murata et al., 1989; Ko et al., 2005; Xu et al., 2006), may also play a role in detoxification. In the GlxI–II pathway, the substrate for GlxI is created by the spontaneous reaction between MG and GSH forming hemithioacetal (HTA). GlxI isomerizes this to S-lactoylGSH (SLG), which is the substrate for GlxII, a hydrolase. The final products are the relatively non-toxic molecule D-lactate and GSH, which is recycled in the cytoplasm. Although a GSH export system (Owens and Hartman, 1986; Pittman et al., 2005) has been identified, there is no evidence for a role in MG detoxification. Protection by the KefGB and KefFC systems is dependent on their role in modulation of the cytoplasmic pH (Ferguson et al., 1995; 1996; 2000; Ferguson and Booth, 1998; MacLean et al., 1998). KefGB and KefFC are structurally related K⁺ efflux systems that are maintained inactive by the binding of GSH and are activated by binding of specific GSH adducts (Elmore et al., 1990; Ferguson et al., 1993; Miller et al., 1997; 2000; Roosild et al., 2009; 2010). Activation leads to rapid K⁺ efflux, which is quantitatively affected by several parameters: external K⁺ concentration, the activity of K⁺ uptake systems, the expression level of KefGB and KefFC and the intracellular concentration of the GSH adduct (Ferguson et al., 1993; 1996; MacLean et al., 1998; McLaggan et al., 2000). K⁺ efflux is accompanied by influx of H⁺ and Na⁺ (Bakker and Mangerich, 1982), but it is the lowering of the cytoplasmic pH that is critical for protection against MG (Ferguson et al., 1995; 2000). Lowering the cytoplasmic pH may slow the reaction of MG with guanine in DNA and with other macromolecules (Krymkiewicz, 1973).

We have previously proposed a model in which GlxI plays the major role in both determining the rate of MG detoxification and in modulation of KefGB activity by the intermediate SLG (Fig. 1) (MacLean et al., 1998). However, the original model could not be fully tested as mutants lacking GlxII could not be created and assays for SLG had not been developed. Thus the model rested on assumptions and essential elements were untested. Here, we report construction and characterization of GlxII mutants (ΔgloB). We integrate measurements of the SLG pools with assays of other parameters involved in MG detoxification and cell survival. In addition to supporting the original model, this comprehensive dataset demonstrate unequivocally the importance for survival of activation of KefGB and the consequent lowering of cytoplasmic pH. In particular we demonstrate that increased activity of the KefGB system can compensate for an impaired capacity to detoxify MG. The data are discussed in terms of the balance between GlxI, GlxII and the K⁺ efflux systems in determining the fate of individual cells.

Results

Modulation of GlxII activity

To assess the importance of the GlxII activity and the activation of KefGB for survival upon exposure to MG we inactivated gloB (see Experimental procedures). Previous attempts to replace the gloB gene with antibiotic resistance cassettes (kanamycin and spectinomycin) were unsuccessful. We considered the possibility that replacement of the entire gloB gene might lead to polar effects with respect to the expression of the two genes on either side of gloB, namely milD and yafS, which are separated by only 71 and 33 bp, respectively, from the gloB ORF (Fig. 2A). The milD gene encodes for a membrane-bound lytic murein transglycosylase, which plays a major role in peptidoglycan expansion and recycling (Scheurwater et al., 2008; Suvorov et al., 2008). The yafS gene is believed to encode an S-adenosyl-L-methionine-dependent methyltransferase, but its physiological role remains unknown. From global array analysis under various growth conditions (GenExpDB, http://genexpdb.ou.edu), it is clear that both these genes are transcribed and thus either or both of these genes may be essential for cell function. Consequently we used a promoter prediction programme (Gama-Castro et al., 2008) (see Supporting information) for the design of the mutagenesis strategy. Based on this analysis, a 454 bp fragment (from 132 to 585) of the gloB structural gene was replaced (Experimental procedures) avoiding the putative promoter sequences for milD and yafS. A short amino-terminal sequence of the GlxII protein (residues 1–43) may be expressed in the mutant strain MJF595 created in this study. However, from the crystal structure, this fragment is unlikely to form an enzymatically active protein as the critical metal and substrate binding sites are located in other regions (Zang et al., 2001; Campos-Bermudez et al., 2007).

The ΔgloB mutant grew at a similar rate to the parent in K₂₃ medium (Fig. S1A) and exhibited no obvious growth phenotype. Thus GlxII is not an essential enzyme.
during normal exponential growth. Some residual activity was detectable (corresponding to ~6% of the parental GlxlII activity), but was close to the analytical limit of the assay. The residual activity was not due to GlxI, as increasing the expression of this enzyme in the ΔgloB mutant did not increase the rate of breakdown of SLG (Table 1). Addition of 0.7 mM MG to both parent and mutant strains, in early exponential phase (OD₆₅₀ = 0.05), caused immediate growth inhibition without recovery over the course of the experiment (Fig. S1A). MG disappeared from the medium in a linear fashion and, as expected, the rate was greatly reduced in ΔgloB cultures (Fig. 2B and 0.444 ± 0.015 μM MG min⁻¹ and 1.155 ± 0.21 μM MG min⁻¹, for mutant and parent respectively). We have previously observed a similar reduction in the capacity to detoxify MG in a ΔgloA null mutant (MacLean et al., 1998). However, cells retain the ability to breakdown MG, but at a much lower rate, which is consistent with the known presence of other enzymes that can metabolize MG (Misra et al., 1995; Misra et al., 1996).

The gloB gene, encoding for the GlxlII enzyme, and its flanking regions were cloned into a moderate copy number vector to create pGlxII (see Experimental procedures). Transformation into E. coli MJF274 (parent strain) led to an approximately 25-fold amplification of GlxlII activity in extracts from mid-exponential grown cells (Table 1). Cells expressing higher levels of GlxlII grew at a similar rate to the parent strain in K₀.2 minimal medium (Fig. S1B). Elevated synthesis of GlxlII did not alter the rate of MG detoxification when cells were incubated with 0.7 mM MG (Fig. 2B). These data are consistent with

**Table 1.** GlxlII activity.

| Strain           | Specific GlxlII activity (U mg⁻¹) |
|------------------|----------------------------------|
| MJF274           | 0.069 ± 0.014                    |
| MJF595           | 0.004 ± 0.011                    |
| MJF274 pGlxII    | 1.789 ± 0.508                    |
| MJF274 pGxlI     | 0.067 ± 0.024                    |

Enzyme activities were performed on three independent cytoplasmic cell extracts from each strain. For each extract the GlxlII activity was measured using two different protein concentrations to ensure that the enzyme was rate-limiting. Activities increased proportionally with protein concentration and were averaged. The mean activity and standard deviation from independent extracts are shown. Strains: MJF274 (parent strain), MJF595 (ΔgloB), MJF274 pGlxII, MJF274 pGxlI (pMJM1).
previous observations that GlxI activity limits the rate of MG detoxification in parental cells (MacLean et al., 1998).

**Inactivation of gloB does not affect cell viability upon MG stress**

Previously, we reported that a ΔgloA mutant, impaired in MG detoxification, exhibits increased sensitivity to MG, which can be explained by the persistence of the electrophile in the growth medium (MacLean et al., 1998). When mutant cultures (either ΔgloA or ΔgloB) were treated with 0.7 mM MG, the concentration remains above the lethal level (~0.6 mM MG) for ~6 h due to the slow rate of detoxification in the absence of the Glx pathway. In contrast, the parent strain detoxifies MG to non-lethal levels within ~2–3 h (at low cell density, OD~0~.~05~). Thus, it was expected that the ΔgloB mutant would exhibit a similar sensitivity to MG as the ΔgloA mutant. However, survival of the ΔgloB mutant during MG exposure was not impaired (Fig. 2C). Surprisingly, overexpression of GlxII increased sensitivity to MG despite having no effect on the rate of detoxification (Fig. 2B and C).

We further addressed if the viability phenotype of the ΔgloB mutant is a reflection of compensatory enzyme activities in vivo. FrmB and YeiG (EC 3.1.2.12) are major components of the formaldehyde detoxification pathway and have been reported to have low level hydrolytic activity against SLG (Gonzalez et al., 2006). The yeiG gene is transcribed constitutively whereas the frmB gene can be induced with formaldehyde (Gonzalez et al., 2006). Strains lacking GlxII and lacking either YeiG (MJF595 ΔyeiG) or FrmB (MJF595 ΔfrmB) were created (Experimental procedures) and cell viability determined during exposure to 0.7 mM MG. The level of survival of both double mutants (ΔgloB−ΔyeiG or ΔgloB−ΔfrmB) was indistinguishable from the ΔgloB mutant (Fig. S2), indicating that these systems do not have a physiologically significant role in MG detoxification.

**K+ efflux systems are hyperactive in a gloB null mutant**

We have previously established that KefGB and KefFC are activated by electrophiles through the formation of GSH adducts (Elmore et al., 1990; Ferguson et al., 1993; 1995; 1997). From the study of a ΔglyA mutant we inferred that SLG was the metabolite activating KefGB during exposure to MG, as the HTA formed by a reversible reaction with GSH in such a mutant was insufficient to activate the K+ efflux system (MacLean et al., 1998). We predicted that a ΔgloB mutant, lacking GlxII activity, would accumulate SLG and thus, KefGB activity should be enhanced; conversely we predicted that overexpression of GlxII should diminish SLG pools and thus lower the rate of K+ efflux. Analysis of K+ efflux patterns in the parent strain MJF274 and the ΔgloB mutant MJF595, using a range of MG concentrations, supports this model. Accurate measurements of K+ efflux require cells to be incubated at higher cell densities than are used for growth and viability measurements (OD~0~.~6~0 ~0.~8~ for efflux assays compared with ~0.~05~ for growth and viability). Consequently, in the parent strain, the MG concentration is continuously declining due to the high rate of detoxification in such conditions (0.7 mM MG falls to ~0.3 mM in 30 min). Control experiments for cell viability and MG detoxification were carried out at high cell density under conditions identical to the measurements of cytoplasmic pH and K+ efflux (Fig. S3A and B). The rate constant for efflux was measured over the first 3 min, a period in which there was minimal lowering of the MG concentration (Fig. S3B). The rate and extent of K+ loss was faster in the ΔgloB strain than in the parent (Fig. 3). This effect was most marked at the lower MG concentrations and thus first order rate constants for the initial K+ efflux were measured at a range of concentrations (Fig. 3C). In the parent strain, the rate of efflux observed at very low MG concentrations (< 200 μM) was not significantly different from the rate of K+ loss in the absence of MG. For higher concentrations the rate constant increased and approached a maximum for concentrations ≥ 3 mM MG (Fig. 3C). In contrast, the ΔgloB mutant exhibited rapid K+ loss even at concentrations as low as 25 μM MG and the rate was not further stimulated by treatment with ≥ 200 μM MG (Fig. 3B and C). In cells overexpressing GlxII MG addition did not stimulate significant K+ efflux (Fig. 3D).

**Intracellular accumulation of SLG upon MG stress**

We developed an LC-MS/MS assay to quantify intracellular GSH and SLG pools to obtain insight into the in vivo dynamics of SLG formation. Cells were grown in K2 minimal medium and GSH and SLG were extracted from cells with formic acid using a silicone oil centrifugation technique (see Experimental procedures). Pools of GSH and SLG were measured over the time period used to measure the rate constant for KefGB and using experimental conditions identical to those for the K+ efflux assays. GSH pools prior to MG addition were identical in the parent and ΔgloB strains within experimental error and no SLG could be detected prior to addition of MG (Table S1). Addition of MG (0.2 mM) caused a rapid increase in SLG, coincident with depletion of GSH, in both ΔgloB and parent; the increase was ~20-fold greater in the mutant than the parent (Fig. 4A). In the parent, SLG pools were below the detection limit of the analytical technique at concentrations < 0.2 mM MG (Fig. 4B). At higher MG concentrations the SLG pool rose rapidly then declined slowly over the period of the assay (Fig. 4A). Pools of SLG in the ΔgloB strain also increased rapidly, even with
only 25 μM MG, achieving ~50% of the maximum value in 10 s, then climbed slowly over the next 2.5 min (Fig. 4C). Despite the absence of GlxII, complete conversion of the GSH pool to SLG was never observed, free GSH was always measurable (~34 μM (Table S1).

High SLG pools were also observed in strains over-expressing GlxI in which the highest SLG level was generally observed at 10 s and decreased thereafter (Table S1), consistent with the higher rates of detoxification observed in this strain (MacLean et al., 1998). SLG levels were significantly higher than in the parent strain (~2.5-fold at 10 s, Fig. 4B and D). However, these did not reach the levels seen in the ΔgloB mutant, reflecting the continuing breakdown. Cells expressing high levels of GlxII did not accumulate significant SLG when incubated with MG (0.1–0.7 mM MG; Table S1).

**Relationship between SLG pool and KefGB activity**

Measurements of the SLG pools and rate constant for K+ efflux under similar conditions allowed the determination of the adduct dependence of KefGB activity. The very
slow MG breakdown in the ΔgloB strain allowed both the SLG pool and KefGB activity to be measured across a wide range of MG concentrations. Parallel measurements were made with the parent strain (Fig. 5A). The rate constant for efflux shows a non-linear dependence on SLG concentration (Fig. 5A). Data from the parent strain were consistent with this relationship (Fig. 5A). When excess MG was supplied (3 mM), which maximally activates KefGB, the rate constant was 0.12 ± 0.02 min⁻¹ and 0.13 ± 0.01 min⁻¹ for the parent and ΔgloB respectively (Fig. 3A and B). Remarkably, strong KefGB activation (to ~25% maximum activity) was observed in the ΔgloB strain even with only 25 μM MG (Fig. 3C), which corresponded to a sixfold excess of GSH over SLG (Table S1). The precise relationship between KefGB activity and SLG pools is expected to be complex as the formation of SLG is accompanied by removal of the KefGB inhibitor, GSH.

Greater cytoplasmic acidification is achieved in a ΔgloB mutant

The activity of the KefGB efflux system ultimately results in acidification of the cytoplasm, which limits the toxicity of MG (Ferguson et al., 1995; Ness and Booth, 1999). We investigated whether the increased activity of KefGB in the ΔgloB cells affected the cytoplasmic pH (pHi) upon MG stress. Experiments were conducted under the same conditions as for K⁺ efflux and SLG pool analysis. Cyto-
plasmic pH fell to a steady state level within 60 s of addition of MG and the change was dependent upon the presence of both KefGB and KefFC. Thus the pH change had different kinetics from K\textsuperscript{+} efflux, which continued for at least 15 min, albeit at progressively slower rates. The discrepancy between the change in pH and K\textsuperscript{+} loss is explained by previous studies that demonstrated that K\textsuperscript{+} efflux is accompanied by entry of both H\textsuperscript{+} and Na\textsuperscript{+} (Bakker and Mangerich, 1982). Cytoplasmic pH changed both as a function of the MG concentration and the strain (Fig. 5B) (a representative dataset showing the kinetics of the pH changes is presented in Fig. S4). There was no consistent difference in the cytoplasmic pH between the parent and the 
\textit{D}gloB strain prior to addition of MG. After MG addition the cytoplasmic pH always fell to a lower value in the mutant, which is the predicted consequence of increased activity of KefGB (Fig. 5B). Overexpression of GlxII (strain MJF595 pGlxII) led to a small drop (~0.1 pH units, data not shown). Overall, a simple correlation was found between the initial rate of loss of viability and the change in the steady state pHi (Fig. 5C).

The role of KefGB in protection against MG

Our failure to observe increased sensitivity to MG in a 
\textit{D}gloB strain (Fig. 2C) might be accounted for by the increased activation of KefGB and sustained lowering of the cytoplasmic pH (Figs 3C and 5C). We therefore constructed a strain lacking both KefGB and KefFC (and GlxII; MJF596, 
\textit{D}gloB, 
\textit{D}kefC, 
\textit{D}kefB). The triple mutant grew at the same rate as the parent strain MJF274 in K\textsubscript{0.2} minimal medium (data not shown) and was similarly impaired in MG detoxification as the 
\textit{gloB} null mutant (0.463 ± 0.063 μM MG min\textsuperscript{−1}; n = 3). Cells were very sensitive to MG (Fig. 6), despite their similar rate of detoxification to the strain lacking only GlxII. No viable cells were observed in the triple mutant after 2 h incubation with

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Survival of $\Delta gloB$ null mutant upon MG stress depends on the activity of K$^+$ efflux systems. Cells that lack the K$^+$ efflux systems KefGB and KefFC, in addition to GloxII, are highly sensitive to MG exposure. Cells from the parent ($\Delta gloA$), $\Delta gloB$ ($\Delta kefB, kefC::Tn10$), and M596 ($\Delta gloB, \Delta kefB, kefC::Tn10$) were grown in K$_0.2$ minimal media, exposed to 0.7 mM MG and viable cells enumerated exactly as for experiments presented in Fig. 2. The mean and standard deviation of three independent experiments is shown.

0.7 mM MG. A mutant lacking both K$^+$ efflux systems (M5976; $\Delta kefB, \Delta kefC$) that retains detoxification was less sensitive to MG than the triple mutant, but cells were killed more rapidly than either the parent strain or the single $\Delta gloB$ mutant (Fig. 6). These data suggest that the efflux systems for K$^+$ are more critical for survival than the detoxification pathway at low cell density.

Discussion

The glyoxalase pathway provides an intrinsic advantage to cells when they are exposed to MG by catalysing the GSH-dependent conversion of MG to d-lactate, which can either be excreted or further metabolized. Mutants lacking GloxI exhibit a high sensitivity to MG; both growth inhibition at low MG concentrations (0.1 mM) and cell death at higher concentrations (0.7 mM) are approximately two fold (MacLean et al., 1998). In most detoxification pathways one would not necessarily look further than the loss of the capacity to remove the toxic molecule for an explanation of the phenotype. However, rapid cell death takes place in a time frame during which the MG concentration remains relatively constant. This is further supported by the $\Delta gloB$ mutant studied here, which does not share the increased sensitivity to MG seen in a $\Delta gloA$ strain despite similar continued exposure to high MG concentrations (Fig. 2). We have previously established that KefGB, but not KefFC, is strongly activated by metabolites derived from MG and GSH. Based on an analysis of the $\Delta gloA$ mutant and of strains possessing either KefGB or KefFC we proposed that KefGB was activated by the product of GloxI, SLG. The survival properties of the mutants studied here become explicable by this linkage and establish that the dominant factor determining survival is not detoxification but activation of the protecting K$^+$ efflux system. Thus, $\Delta gloA$ (GloxI) and $\Delta gloB$ (GloxII) mutants exhibit almost identical rates of MG detoxification that are approximately threefold lower than observed in the parent (Fig. 2B, MacLean et al., 1998). However, in a GloxI mutant the only adduct formed is HTA, whereas in a GloxII mutant, SLG accumulates to high levels and the GSH pool is severely depleted (Fig. 4A; Table S1). GSH is an inhibitor of KefGB and HTA is only a weak activator, which would result in only limited activation of KefGB in a $\Delta gloA$ strain. In contrast, the enhanced pools of SLG and depletion of GSH in a GloxII mutant create optimal conditions for activation of KefGB (Fig. 3B and C) and result in a corresponding greater acidification of the cytoplasmic pH (Fig. 5B). Support for this model comes from our studies on the effect of the overexpression of GloxI or GloxII. The former stimulates detoxification, which suggests that this enzyme is limiting for the pathway. However, the stimulation is not proportional to the increase in enzyme activity, which is consistent with a fairly small gap between the relative activities of GloxI and GloxII, such that as GloxI activity is increased, GloxII becomes the limiting factor. Under these circumstances SLG accumulates (Table S1), with the effect of stimulating KefGB activity, which explains the dramatically increased protection against MG by overexpression of GloxI (MacLean et al., 1998). In contrast, overexpression of GloxII does not modify the detoxification rate, but sensitizes cells to MG (Fig. 2C). An increase in GloxII, in the context of a low but constant level of GloxI, depletes the SLG pool (Table S1) resulting in limited activation of KefGB and hence only moderate protection. Finally, the combination of mutations that eliminate KefGB, KefFC and GloxII renders cells as sensitive to MG as if they had a $\Delta gloA$ mutation (Fig. 6).

The relationship between the magnitude of the SLG pool, KefGB activity and pH$_i$ is complex, but can be understood from the properties of the systems and the cell itself. The buffering capacity of the cytoplasm is at its lowest value around cytoplasmic pH values in the range pH 7–8 (Booth, 1985). Thus the cell is simultaneously at its most sensitive to perturbation of the environment due to limited buffering capacity, but also has the greatest potential to modulate cytoplasmic pH through changes in the balance of proton entry and efflux pathways. Previous work established that K$^+$ efflux from E. coli is compensated by the entry of both H$^+$ and Na$^+$ in a ratio of approximately 1K$^+~0.4H^+$ and 0.6Na$^+$ (Bakker and Mangerich, 1982). The extremely rapid acidification of the cytoplasm upon activation of KefGB can be explained by initial rapid K$^+$-linked H$^+$ movements that are subsequently compensated by the
reversibility of the Na’/H’ antiports (Padan et al., 2001) leading to exchange of cytoplasmic H’ for external Na’. Thus, the pH would be stabilized at a value set by the intrinsic properties of the KefGB system and the Na’/H’ antiports (NhaB and NhaA) (Dover and Padan, 2001; Padan et al., 2001). Our recent work (Roosild et al., 2010) shows that a single binding site on KefGB is shared by GSH and glutathione adducts. Activation of KefGB by MG requires displacement of GSH and binding of SLG. The cytoplasmic pool of GSH is suggested to be ~20 mM (McLaggan et al., 2000; Fahey, 2001; Bennett et al., 2009). We observed that activation of KefGB in a ΔgloB strain requires only low concentrations of SLG, even in the presence of a sixfold excess of GSH (Figs 4A and 5A). Even in the parental strain, SLG is always at a lower concentration than GSH and consequently, the activation of KefGB is not maximal at the normal levels of abundance of GlxI and GlxII. Thus, the change in cytoplasmic pH is constrained by the relative abundance of GlxI and GlxII activities and the concentration of MG.

The data presented here point to an important relationship between the intrinsic activity of GlxI, GlxII and KefGB. The effects of GlxI overexpression indicate that although the enzyme is, on a population basis, limiting for the rate of detoxification. A 30-fold increase in GlxI activity produces only a twofold enhancement of the detoxification rate (MacLean et al., 1998) (see also Fig. S3B). From this we infer that GlxI and GlxII activities in cells are similar, such that large increases in GlxI cannot be manifested as increased detoxification rates due to the limitation that is imposed by GlxII activity. Given that most detoxification enzymes are expressed at low levels in the cell there is considerable scope for cell-to-cell variation in enzyme level. GlxI and GlxII are encoded by separate unlinked genes and there is no co-ordination of their expression. Consequently, in each cell their abundance should vary independently. We have calculated the abundance (in cells grown to mid-exponential phase in minimal medium) to be ~130 GlxI and ~1500 GlxII molecules per cell (see Supporting information). Stochastic variation of protein abundance will have a greater impact on GlxII and thus some cells will have a balance that favours SLG accumulation (high GlxII: GlxI > GlxII) whereas others (low GlxII: GlxII > GlxII) will have lower cytoplasmic pools of this adduct. Thus, on an individual cell basis, some cells will experience greater protection than others. The independence of the expression of KefGB from the detoxification enzymes creates a further dimension of variability that is superimposed on the modulation of SLG pools. This means that some cells may gain further protection; the optimal solution would be simultaneous enhanced levels of GlxII and KefGB, coupled with low activity of GlxII. In this way the cell will experience maximum protection coupled with detoxification.

In this study we have defined the relationship between sensitivity to MG and the relative activities of the major detoxification pathway, GlxI–II, and the KefGB K+ efflux system. The study demonstrates the benefits to the cell of linking an intermediate in detoxification to the activation of the protective K+ efflux system. Small changes in detoxification enzymes determine the maximum activity of KefGB. It is perhaps counterintuitive that cells possess protective capacity that is not always fully utilized. At low cell density detoxification capacity is secondary to ion channel activity in determining single cell fate. Conversely at high cell densities detoxification capacity is the primary determinant of cell survival. Stochastic distribution of the proteins between cells in a population ensures that a few cells are highly protected and survive exposure to MG. In addition, it seems plausible that avoiding maximum stimulation of KefGB in all cells prevents excessive acidification that might be itself detrimental. The paradox is that in wild-type populations the majority (99.9%) of cells are terminally damaged (i.e. unable to form colonies after dilution and plating on fresh medium) but continue to contribute to MG removal. Thus, the survivors are aided by the dead and dying cells.

Experimental procedures

Strains and plasmids

All experiments were performed with E. coli K-12 derivative strains and are listed in Table 2. See further below for details of strains and plasmids created in this study.

Growth media

For physiological assays cells were grown either in K12 minimal medium containing ~0.2 mM K+ or K15 minimal medium containing ~115 mM K+ (Epstein and Kim, 1971) depending on the experimental design. Both media were supplemented with 0.2% (w/v) glucose, 0.001% (w/v) thiamine, 0.4 mM MgSO4 and 6 μM (NH4)2SO4·FeSO4. LK complex medium (Rowland et al., 1985) was used for cell growth for DNA manipulations. In the case of strains carrying an antibiotic resistance marker, no antibiotics were used in the growth media.

Cloning of the gloB gene

The gloB gene was PCR-amplified using whole cells of MJF274 as a template. Primers GloBI (5′-CAACCA GCGTCGACTGTAC) and GloBII (5′-GGTATCACCCAGTGG CAACCA GCGTCGACTGTAC) were designed 1.1 kb downstream and 1 kb upstream of gloB respectively. The primers contained HincII and BamHI
restriction sites respectively (underlined). The amplified 2.8 kb product was digested with HindIII and BamHI restriction enzymes. The cloning vector pHG165 was digested with HindIII, followed by treatment with Klenow enzyme to fill the HindIII site. Subsequently, the vector was digested with BamHI. The PCR product was ligated into the vector to create plasmid pGlxII from which the gloB gene is expressed from its native promoter.

**Construction of gloB null mutants**

The gloB gene was disrupted with a chloramphenicol resistance cassette (Cm<sup>n</sup>) by homologous recombination mediated by λ-phage functions (recombineering). Recombineering primers gloB KO-A (5’-TGCGCGTCTATTATTATCAAG ATACGGGTAGTTTATATTATTATTTGGCCGACCGCAACAA TAGACATAAGCG) and gloB KO-B (5’-GATCCCGGAGCGCA GAGGCGGATATCAAGCAGCTCGCCGCAATAACTG ATAAATAATCTTGTTCGTCCC) contained homologous sequences to the 3’ and 5’ of the gloB gene respectively. The incorporation of the Cm<sup>n</sup> cassette was predicted to create a fused ORF with the remaining 5’ gloB sequence. Therefore, a double stop codon was incorporated into primer gloB KO-B (underlined) to prevent possible expression of a fusion protein driven by a gloB promoter. A 454 bp fragment (from 132 to 585) of the structural gene, where 1 is the first bp of the start codon, was replaced by the Cm<sup>n</sup> cassette. Hence a short sequence of the GloXII protein may be expressed. The Cm<sup>n</sup> cassette was amplified by colony-PCR from a strain carrying plasmid pGlxII. The amplified GloXII cassette was cloned and is transcribed from its own promoter.

| Strain   | Genotype | Source/reference |
|----------|----------|------------------|
| MUF274   | F. thi, rha, lacZ, kdpABC5, lacI, trkD1 | Ferguson et al. (1993) |
| MUF276   | MUF274, kefB, kefC::Tn10 | Ferguson et al. (1993) |
| MUF595   | MUF274 ΔgloB::cm | This study |
| MUF596   | MUF276 ΔgloB::cm | This study |
| MUF625   | MUF595 ΔyeG::kan | This study |
| MUF626   | MUF595 ΔfrmB::kan | This study |
| DY330    | W3110 ∆lacU169gal490 pglΔ8 λcl857 λ(cro-bioA) | Yu et al. (2000) |
| JW2141   | BW25113 ∆yeG::kan | Baba et al. (2006) |
| JW0346   | BW25113 ∆frmB::kan | Baba et al. (2006) |
| Plasmid  | Description | Source/reference |
| pHG165   | pBR322 copy number derivative of pUC8 | Stewart et al. (1986) |
| pMJM1    | A pHG165 derivative into which gloA was cloned and is transcribed from its own promoter. | MacLean et al. (1998) |
| pGixII   | A pHG165 derivative into which gloB was cloned and is transcribed from its own promoter. | This study |
Interestingly, we observed some differences in the transfer of the gloB null mutation into different E. coli strains by P1 transduction. The gloB<->cm mutation, initially created in strain DY330, could be transduced into strain MJF274, as described above, and transductants were obtained after overnight incubation (~16 h). In contrast, upon transfer of the mutation into strain MG1655, transductants were only evident after prolonged incubation (~40 h) despite similar growth rates of both parent strains. Notably, upon purification, the MG1655 derivative strain grew at a similar rate as the parent strain and had no obvious phenotype (data not shown) possibly indicating the acquisition of a secondary mutation allowing cells to recover normal growth.

**K⁺ efflux assays**

K⁺ efflux from cells was measured as described previously (Elmore et al., 1990; Ferguson et al., 1993). Briefly, cells were grown to late logarithmic phase (OD_{650} ~0.8) in K_{115} medium, collected by filtration onto a cellulose acetate membrane (0.45 μm), washed with K₀ buffer containing ~5 mM (K₀ buffer) and suspended in K₀ buffer. Cells were rapidly transferred into thermally insulated glass pots and kept at 37°C under continuous stirring. Samples (1 ml) were taken at various time points, cells pelleted in a microcentrifuge at 14 000 r.p.m. for 30 s, the supernatant quickly aspirated and the K⁺ content of the cells determined by flame photometry after lysis by boiling in distilled water. MG (Sigma, M0252) was added from stock solutions to the test suspension 2 min after suspending cells in K₀ buffer. The first order rate constants for K⁺ efflux (k) were calculated by transforming the values of K⁺ levels to the natural logarithm and by determining the slope of the decline in the linear range after addition of MG. For illustration purposes the K⁺ levels were normalized to the value at t_min, defined as 100%.

**Cell viability and MG detoxification assays**

Overnight cultures were grown in K_{0.2} medium and diluted into fresh, pre-warmed K_{0.2} medium to an OD_{650} of ~0.05. Cells were grown to mid-exponential phase (OD_{650} ~0.4) and diluted 10-fold into pre-warmed K_{0.2} medium also containing MG. Samples were taken at various time points and cell viability and MG detoxification assays performed as described previously (Ferguson et al., 1993; Totemeyer et al., 1996). Viable cells were recovered on solid K_{0.2} media plates.

**Preparation of cytoplasmic cell extracts and Glix II enzyme assays**

Cells were grown in K_{0.2} medium to mid-exponential phase (OD_{650} ~0.4) exactly as for the assays above; however, they were harvested at this point by centrifugation at 4300 g. Cells were washed, suspended in 50 mM potassium phosphate buffer (pH 6.8) and disrupted by two passages through a French press at 18 000 Psi. Bulk cell debris and membrane fractions were removed by centrifugation at 4300 g and subsequent centrifugation at 110 000 g. Cytoplasmic cell extracts were stored at ~20°C until enzyme assays were performed. Protein quantification was performed using the Lowry assay (Lowry et al., 1951). Glix II activities were measured as a modification of a previously described method (Racker, 1951; Oray and Norton, 1982), in which SLG hydrolysis is measured by the decrease in A_{240}. Enzyme assays were performed in 50 mM potassium phosphate buffer (pH 6.6) at 37°C using a Shimadzu UV 2101PC spectrophotometer. The reaction mixture contained 1 mM SLG (Sigma, L7140) in a total volume of 0.4 ml, using a 0.1 cm path length spectrophotometer cuvette. Enzyme activity was expressed as units per cytoplasmic cell protein (U mg⁻¹) using a molar extinction coefficient of 3060 M⁻¹ cm⁻¹ (Racker, 1951), where 1 unit is defined as the amount of enzyme catalysing the formation of 1 μmol min⁻¹ SLG. Enzyme activities were determined using two different enzyme concentrations to ensure that the enzyme was rate-limiting.

**Determination of intracellular GSH and SLG levels by LC-MS/MS**

The choice of the experimental design to extract the metabolites from the cells was guided by the need to correlate SLG levels directly with K⁺ efflux. Therefore, cells were grown and treated as in K⁺ efflux experiments as described above except that cells were grown in K_{0.2} medium. After suspension of cells in K₀ buffer 1 ml samples were taken at various time points before and after exposure to MG and transferred into previously prepared microcentrifuge tubes. Microcentrifuge tubes contained 40 μl 2.5 M formic acid (Sigma, 251364) with 50 μM Glu-Glu (Sigma, G3640, as an internal standard for LC-MS/MS analysis), overlaid with 500 μl silicone oil mixture prepared from silicone oils of different densities (AR20; Fluka, 10836; AP100; Fluka, 10838; proportion of 3:2). The sample tubes were centrifuged at 14 000 r.p.m. for 30 s. Cells that passed through the silicone into the formic acid were permeabilized and thus all cell reactions ceased immediately. Medium and silicone oil were removed by vacuum aspiration and the formic acid, with the cell debris, was transferred into fresh microcentrifuge tubes. The samples were centrifuged at 14 000 r.p.m. for 15 min at 4°C, the supernatants removed to separate tubes and stored at ~20°C. Subsequently, samples were analysed by LC-MS/MS using quantification based on standard curves for GSH (Sigma, G6529) and SLG (Sigma, L7140) prepared on the same day as the cellular samples by adding appropriate volumes from frozen stocks to the formic acid/Glu-Glu extraction solution. The LC-MS/MS was performed using a Thermo Surveyor-TSQ Quantum system with electrospray ionization in the positive ion mode. A Stability BSC 17 (5 μ) column (150 x 2 mm) was used and the analytes eluted with a mobile phase comprising: 50% 7.5 mM ammonium formate, pH 2.6 (formic acid) and 50% acetonitrile at a flow rate of 0.2 ml min⁻¹. The column was maintained at 45°C. Electrospray ionization conditions were as follows: spray voltage 4 kV, sheath gas pressure 60, auxiliary gas 0 and capillary temperature 375°C. Detection was carried out in SRM mode at a collision pressure of 1.4 and a collision energy of 13 V using the following SRM transitions: GSH m/z 308 – m/z 179, SLG m/z 380 – m/z 233 and Glu-Glu (internal standard) m/z 277 – 241. Quantification was performed using Xcalibur software. All samples and standards were diluted 1:100 with...
water prior to injection (1 μl) and were maintained at 4°C in the autosampler. It was not possible to resolve SLG and GSH chromatographically; however, parent masses and fragment masses were suitably different to allow discrimination by the subsequent MS. Control experiments with a gloA null mutant verified that the LC-MS/MS assay did not detect the isomer of SLG, namely HTA. HTA is the product of the chemical equilibrium between MG and GSH (Fig. 1). The equilibrium of this reaction is in favour of HTA, which is then converted to SLG by the action of the GlxI enzyme. Note that, in this assay, measured GSH levels do not necessarily reflect in vivo GSH levels during the MG detoxification process because of the chemical equilibrium with HTA. Measured GSH levels will be a composite of actual GSH levels and GSH that was conjugated as HTA at the time of cytoplasmic extraction. The HTA molecule is unstable upon disturbance of the equilibrium with GSH + MG, i.e., upon dilution of the cytoplasmic cell volume in formic acid the equilibrium is driven back to GSH and MG. Furthermore, metabolite concentrations presented in this study are the levels as quantified in the extraction volume (40 μl formic acid). An approximation of intracellular concentrations can be derived from the relationship between OD650 and cytoplasmic volume [1 ml cell culture at concentrations can be derived from the relationship between OD650 and cytoplasmic volume [1 ml cell culture at OD650 = 2 × 0.64 μl (8 × 10⁸ cells), and a concentration of 100 μM in the extraction volume equates to an intracellular concentration of ~6.35 mM.

Measurement of intracellular pH

The magnitude of the pH gradient was estimated from the distribution of a weak acid, and pH, (internal pH) calculated from knowledge of pH, (external pH). Cells were grown in K0.2 minimal medium until they reached an OD650 ~0.8. The cell suspension was transferred into two thermally insulated glass pots (37°C) and 14C benzoic acid (4.5 μM final concentration; specific activity 0.1 μCi ml⁻¹) and 3H-water (specific activity 1 μCi ml⁻¹), as marker of extracellular water, were added to the cultures (Kroll and Booth, 1981). After 5 min incubation, 1 ml samples were taken at timed intervals and the cells and supernatant separated by centrifugation (14 000 r.p.m. for 20 s). For each sample, 100 μl of the supernatant was transferred to a scintillation vial containing 200 μl of cell suspension that had not been treated with radioactivity; the remaining supernatant was aspirated from the cell pellet and discarded. Cell pellets were suspended in 200 μl K0.2 buffer containing 0.2% glucose and the suspension transferred into a scintillation vial containing 100 μl of the same buffer. Samples of supernatant and pellet were counted for radioactivity on a preset 3H/14C program of a Tri-Carb 2100 TR liquid scintillation analyser. The pH gradient and subsequently the pH were calculated as described previously (Booth et al., 1979).

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