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Methylglyoxal resistance in *Bacillus subtilis*: Contributions of bacillithiol-dependent and independent pathways

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Summary

Methylglyoxal (MG) is a toxic byproduct of glycolysis that damages DNA and proteins ultimately leading to cell death. Protection from MG is often conferred by a glutathione-dependent glyoxalase pathway. However, glutathione is absent from the low-GC Gram-positive Firmicutes, such as *Bacillus subtilis*. The identification of bacillithiol (BSH) as the major low molecular weight thiol in the Firmicutes raises the possibility that BSH is involved in MG detoxification. Here, we demonstrate that MG can rapidly and specifically deplete BSH in cells, and we identify both BSH-dependent and BSH-independent MG resistance pathways. The BSH-dependent pathway utilizes glyoxalase I (GlxA, formerly YwbC) and glyoxalase II (GlxB, formerly YurT) to convert MG to D-lactate. The critical step in this pathway is the activation of the KhtSTU K^+ efflux pump by the S-lactoyl-BSH intermediate, which leads to cytoplasmic acidification. We show that cytoplasmic acidification is both necessary and sufficient for maximal protection from MG. Two additional MG detoxification pathways operate independent of BSH. The first involves three enzymes (YdeA, YraA and YfkM) which are predicted to be homologues of glyoxalase III that converts MG to D-lactate, and the second involves YhdN, previously shown to be a broad specificity aldo-keto reductase that converts MG to acetol.

Keywords

Bacillus subtilis; bacillithiol; methylglyoxal; glyoxalase; electrophile

Introduction

Methylglyoxal (MG) is a toxic, endogenous byproduct of glycolysis (Ackerman *et al.*, 1974). It is synthesized by methylglyoxal synthase (MGS) under conditions of carbon excess or phosphate limitation, which lead to an imbalance between the rate of carbon acquisition and the lower segment of glycolysis (Booth *et al.*, 2003). The main role of MGS is to restore inorganic phosphate levels. Bacteria can also encounter MG in the environment (Booth *et al.*, 2003; Landmann *et al.*, 2011). MG has been found in many food and beverages and thus members of the gut microbiota may be regularly exposed to MG (Griffith and Hammond, 1989). In addition, MG is produced by macrophages as part of the response to invasion by pathogenic bacteria. As an electrophile, MG can modify guanine bases in DNA, leading to DNA damage and an increased rate of mutation (Papoulis *et al.*, 1995). Accumulation of MG is also associated with an increase in the rate of "persister" cell formation in *E. coli*

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(Girgis *et al.*, 2012). Furthermore, MG can react with arginine, lysine, and cysteine residues in proteins resulting in protein inactivation (Leoncini *et al.*, 1980).

The major mechanism of protection from MG in *E. coli* is dependent on the highly conserved glyoxalase system and the low molecular weight (LMW) thiol glutathione (GSH) (Ferguson and Booth, 1998). Exposure to MG leads to the spontaneous formation of the GSH-hemithioacetal (HTA) adduct. Glyoxalase I (GlxI) catalyzes the formation of S-lactoyl glutathione (S-lactoyl-GSH) from HTA (MacLean *et al.*, 1998). Recent reports in *E. coli* suggest that glyoxalase I expression is upregulated in response to MG as a result of readthrough transcription from the *nemRA* operon (Lee *et al.*, 2013; Ozyamak *et al.*, 2013). The S-lactoyl-GSH intermediate is critical to protection from MG stress as it is required for the full activation of the KefGB K^+ efflux pump (Ozyamak *et al.*, 2010). The H^+ influx that accompanies KefGB-mediated K^+ efflux leads to cytoplasmic acidification, and this acidification is sufficient to confer resistance to MG exposure (Ferguson *et al.*, 1993). Finally, glyoxalase II converts S-lactoyl-GSH to D-lactate and regenerates GSH (Ozyamak *et al.*, 2010). In addition, GSH independent pathways have been identified in *E. coli*. HchA (glyoxalase III), originally identified as a heat shock protein with weak chaperone activity, converts MG directly to D-lactate (Subedi *et al.*, 2011). Also, a number of low specificity aldo-keto reductases may make minor contributions to MG resistance (Ko *et al.*, 2005).

Bacillus subtilis mounts a transcriptional response to alleviate the cell damage caused by MG exposure (Nguyen *et al.*, 2009). For example, the SOS regulon is induced, possibly in response to the modification of DNA by MG. Depletion of the LMW thiol pool by MG leads to an imbalance in thiol-redox homeostasis. As a result, the Spx (thiol homeostasis), CtsR (misfolded protein response), and CymR (cysteine formation) regulons are induced. The Spx regulon includes *ytpQ*, which has been implicated in both iron homeostasis and prevention of oxidative protein damage after exposure to MG (Zuber *et al.*, 2011).

Until recently, the major LMW thiol in the low-GC Gram-positive bacteria (Firmicutes) was assumed to be cysteine or coenzyme A (CoA). In 2007, bacillithiol (BSH) was identified as the major LMW thiol in *B. subtilis*. We originally detected BSH in mixed disulfides formed by the organic peroxide sensing transcription regulator, OhrR, under conditions of oxidative stress (Lee *et al.*, 2007). Since the identification of BSH in *B. subtilis*, a bioinformatic survey suggests that BSH is widely distributed among the low-GC Gram-positive bacteria, whose members include *B. anthracis*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Newton *et al.*, 2009). Structurally, BSH is comprised of L-cysteine linked to a glucosamine and malic acid (Newton *et al.*, 2009). BSH is similar to another alternative LMW thiol found in Gram positive bacteria, mycothiol, but is considerably different from the GSH tripeptide. Organisms containing BSH contain BSH-dependent enzymes analogous to their GSH-dependent counterparts. To date, the best characterized is the BSH-dependent thiol transferase FosB involved in resistance to fosfomycin (Gaballa *et al.*, 2010; Roberts *et al.*, 2013; Thompson *et al.*, 2013), which is structurally and functionally analogous to its GSH-dependent counterpart, FosA (Bernat *et al.*, 1997).

We postulated that BSH serves a protective function against MG stress in a manner functionally analogous to GSH. The first hint came from the observation that the gene encoding methylglyoxal synthase (MGS), *mgsA*, shares an operon with the genes encoding the first two enzymes in the BSH biosynthetic pathway, *bshA* and *bshB1* (Gaballa *et al.*, 2010). Furthermore, the genes required for BSH synthesis are members of the Spx regulon (Rochat *et al.*, 2012; Gaballa *et al.*, 2013), which is induced in response to MG (Nguyen *et al.*, 2009). Consistent with a role in MG resistance, strains lacking BSH are more sensitive to exogenous MG than wild-type (Gaballa *et al.*, 2010).

In this work, we identify both BSH-dependent and BSH-independent pathways for MG detoxification in *B. subtilis* (Fig. 1). Our genetic studies reveal two BSH-dependent enzymes, glyoxalase I (YwbC; renamed GlxA) and glyoxalase II (YurT; renamed GlxB), that convert MG to D-lactate. The critical step in this pathway is the activation of the KhtSTU K^+ efflux pump by the production of S-lactoyl-BSH, which leads to cytoplasmic acidification. In addition, we have identified two BSH-independent MG detoxification pathways. The first involves YdeA, YraA, and YfkM, structural homologs of *E. coli* glyoxalase III (HchA), and the second involves YhdN, a broad specificity aldo-keto reductase that converts MG to acetol.

Results and Discussion

Determination of the BSH-dependent methylglyoxal detoxification pathway

Previous studies showed that BSH null cells are more sensitive to MG than wild-type cells, consistent with a possible role for BSH in enzymatic MG detoxification (Gaballa *et al.*, 2010). Given that the major mechanism of MG detoxification in many organisms relies on the glyoxalase I/II pathway, we searched the *B. subtilis* genome for putative glyoxalase enzymes. We previously identified *ywbC* and *yurT* as candidates for glyoxalase I and glyoxalase II, respectively (Nguyen *et al.*, 2009). Based on the results reported herein, we rename YwbC to GlxA (glyoxalase A) and YurT to GlxB (glyoxalase B) to reflect their function in MG resistance.

Since GlxA is predicted to catalyze the conversion of the hemithioacetal (HTA) adduct to S-lactoyl-BSH in the first step in MG detoxification, we predicted that a *glxA* mutant would be more sensitive to MG than wild-type. Indeed, in a *glxA* mutant we observe an increase in MG sensitivity equal to what is observed with a BSH null strain (*bshC*) by disk diffusion assays (Fig. 2). MG sensitivity of the *glxA bshC* double mutant was comparable to the *glxA* or *bshC* single mutants, suggesting that GlxA and BSH are in the same pathway. This supports the assignment of GlxA as a novel, BSH-dependent ortholog of glyoxalase I. Moreover, these results indicate that GlxA is required for the major BSH-dependent pathway for MG resistance.

We next tested GlxB, the putative glyoxalase II enzyme, for a role in MG resistance. Glyoxalase II converts S-lactoyl-BSH to D-lactate that can either enter the TCA cycle or be excreted from the cell. In contrast with the *glxA* mutant, a *glxB* mutant has a small increase in MG resistance (Fig. 2). This increase in resistance may be due to an accumulation of the S-lactoyl intermediate, which by analogy with the results from *E. coli* (Ozyamak *et al.*, 2010), could result in greater cytoplasmic acidification in response to MG. Consistent with this hypothesis, the observed increase in MG resistance in the *glxB* mutant is dependent on BSH: the MG sensitivity of a *glxB bshC* double mutant is comparable to the *bshC* single mutant (Fig. 2).

In *E. coli*, production of S-lactoyl-GSH by glyoxalase I leads to activation of the protective KefGB and KefFC K^+ efflux systems (Ferguson *et al.*, 1993). Activation of the KefGB and KefFC systems leads to cytoplasmic acidification, which is critical for survival of MG stress. We sought to identify candidates for K^+ efflux systems that may serve a similar function in *B. subtilis*. *B. subtilis* possesses a K^+ efflux pump encoded by the *khtST yhaU* operon that has been well characterized *in vitro* (Fujisawa *et al.*, 2007). YhaU is a member of the cation/anion antiporter 2 (CPA2) family of transporters, whose members also include KefGB and KefFC, while KhtS and KhtT are ancillary proteins that modulate YhaU function (Fujisawa and Wada, 2004). We propose renaming YhaU to KhtU, adopting a similar naming convention to its ancillary proteins KhtS and KhtT.

We used a genetic approach to determine if KhtU plays a role in MG resistance. We observed that the *khtU* mutant is more sensitive to MG than wild-type (Fig. 2), consistent with the hypothesis that the KhtU K^+ efflux pump is involved in MG resistance. Moreover, the *khtU bshC* double mutant is no more sensitive than the *khtU* single mutant. This suggests that activation of KhtU relies on BSH, and further suggests that this activation is the dominant, or perhaps only, contribution of BSH to MG resistance.

The glyoxalase I/II pathway may contribute to MG resistance by either or both of two mechanisms. First, the conversion of MG to S-lactoyl-BSH may activate the KhtU K^+ efflux pump leading to cytoplasmic acidification. Second, the enzymatic removal of MG by GlxA may detoxify the cytosol. To address the relative contributions of these pathways, we measured the MG sensitivity of a *khtU glxB* mutant. This mutant is predicted to accumulate S-lactoyl-BSH, but is missing the KhtU K^+ efflux system. If the conversion of MG to S-lactoyl-BSH is sufficient for protection, we predict that the *khtU glxB* double mutant would be as resistant to MG as the *glxB* single mutant. However, we observed that the *khtU glxB* double mutant is as sensitive to MG as the *khtU* single mutant, suggesting that conversion of MG to S-lactoyl-BSH is insufficient for MG resistance under these conditions (Fig. 2).

To further investigate the role of the BSH-dependent pathway in MG detoxification, we measured intracellular BSH levels after MG treatment. In wild-type cells, BSH concentrations increase at higher cell densities with typically 2–4 μmol per gm dry weight during mid-logarithmic phase (corresponding to ~0.7–1.3 mM cytosolic BSH; see Experimental Procedures) (Fig. 3). BSH levels are rapidly depleted (~90% lower than untreated cells) upon exposure to 1 mM MG and this depletion occurs within 10–15 min. of treatment. Similar results are seen in a *bshB2* mutant that lacks an enzyme that can deacetylate the BSH precursor GlcNAc-Mal (Gaballa *et al.*, 2010), and may also serve as a bacillithiol-S-conjugate amidase (BCA) (Fang *et al.*, 2013). The *bshB2* null strain still produces near wild-type levels of BSH (Fig. 3B), due to the activity of the BshB1 deacetylase, as shown previously (Gaballa *et al.*, 2010). Over the course of several hours, BSH recovers to levels comparable to those in the untreated cells at the same growth phase. We hypothesized that the recovery of BSH might be due to the activity of GlxB, which converts S-lactoyl-BSH to D-lactate, thereby recycling BSH. Consistent with this hypothesis, BSH levels did not recover after MG treatment in the *glxB* mutant strain over the time course of the experiment (Fig. 3D). In cells lacking GlxA, BSH levels also recover after MG addition (Fig. 3C). In the absence of GlxA, the product of the spontaneous reaction between BSH and MG (the HTA adduct) cannot be converted to S-lactoyl-BSH. Since the formation of the HTA adduct is reversible, we propose that the observed recovery of BSH in the *glxA* mutant is due to alternative MG detoxification pathways as described below.

Methylglyoxal induces BSH-dependent cytoplasmic acidification

Studies from the Booth lab demonstrated that activation of the Kef K^+ efflux systems in response to MG leads to cytoplasmic acidification, which is required for MG resistance (Ferguson *et al.*, 1993). To determine if KhtSTU leads to cytoplasmic acidification, we monitored the intracellular pH (pHi) of cells treated with MG using the pH sensitive GFP reporter pGFPmut3 (Kitko *et al.*, 2009). In the absence of MG, wild-type, *bshCglxB* and *khtU* strains all had an intracellular pH (pHi) near 7.7 (Fig. 4), consistent with previously reported values (Kitko *et al.*, 2009). Upon treatment with 1.5 mM MG, the pHi of the wild-type decreased ~0.4 pH units to ~7.3 (Fig. 4A). Cytoplasmic acidification is dependent on both BSH and KhtU, since the pHi of *bshC* and *khtU* mutants remained at 7.7 even after MG treatment (Fig. 4B, D). We also examined the pHi of the *glxB* mutant as previous studies in *E. coli* have shown that glyoxalase II mutants have a greater pHi decrease in response to MG due to an accumulation of S-lactoyl-GSH (Ozyamak *et al.*, 2010). However, we did not

observe a similarly increased response (Fig. 4C). These data support a model in which MG results in cytoplasmic acidification mediated by the KhtU K^+ efflux pump. Since acidification is dependent on BSH (but not on GlxB), we suggest that S-lactoyl-BSH may serve to gate the KhtU K^+ efflux system just as S-lactoyl-GSH does for *E. coli*.

Maintenance of appropriate intracellular pH and cell turgor pressure by regulation of K^+ levels is critical to cell viability (Booth, 1985). Both *B. subtilis* KhtU and the *E. coli* Kef channel belong to a family of cation/proton antiporters (CPA2) that is characterized by the ability to couple cation efflux with proton influx and the presence of a K^+ transport nucleotide binding (KTN) domain, which is responsible for the cytoplasmic regulation of K^+ channels and transport. In addition to activation by S-lactoyl-GSH, GSH itself inhibits Kef function. In the absence of GSH, the Kef channel has an intermediate level of activity between the fully closed and open states (Elmore *et al.*, 1990). Structural and biochemical studies have revealed that GSH binds to a cleft formed by the two KTN domains of the KefFC dimer rendering it inactive by stabilizing an interaction between the two monomers (Roosild *et al.*, 2010). The structure of KefFC bound to a GSH adduct suggests that S-lactoyl-GSH (leading to activation) and GSH (leading to inhibition) likely compete for the same binding site.

Our data suggest that BSH may serve a similar function in the inhibition of KhtU since a *khtU* mutant is more sensitive to MG than a BSH null strain. In the absence of BSH, the KhtU channel may be partially active prior to MG addition offering a small level of additional protection from MG through a slight decrease in cytoplasmic pH, whereas a strain lacking KhtU is completely unable to respond to MG. However, we were unable to observe differences in the pHi of a BSH null strain compared to wild-type. Further studies on the possible function of KhtU as a BSH-gated K^+ efflux channel should provide additional insight into the regulation of intracellular K^+ levels.

Cytoplasmic acidification is sufficient for protection of cells from MG even in the absence of BSH

We next sought to determine if cytoplasmic acidification is sufficient to protect *B. subtilis* from MG. To address this question, we treated cells with a concentration of sodium benzoate determined, in preliminary experiments, to cause a ~0.4 unit drop in pH (Kitko *et al.*, 2009, and data not shown). Mid-logarithmic phase cells were treated with 3 mM MG for 5 min before addition of 30 mM sodium benzoate. Upon MG addition, we observed a 2-fold decrease in cell viability (colony forming units) for wild-type cells and a 4–10 fold decrease in viability for *bshC* and *khtU* mutants, which provides further evidence for the role of BSH and KhtU in MG resistance (Fig. 5A, B, C). Addition of sodium benzoate alone had no effect on cell viability. When sodium benzoate was added 5 min. after MG addition, wild-type cells were protected from MG toxicity (Fig. 5A). Importantly, *bshC* and *khtU* strains, which are unable to decrease intracellular pH in response to MG, were also protected from MG by addition of sodium benzoate (Fig. 5B, C). These data support the inference noted above that cytoplasmic acidification is the primary pathway of BSH-dependent MG resistance.

Cytoplasmic acidification is the dominant role of BSH in MG resistance

Protection from MG by the glyoxalase system can be achieved by two mechanisms: (1) removal of MG by formation of the hemithioacetal and conversion to D-lactate by glyoxalase I and II and (2) cytoplasmic acidification through activation of K^+ efflux channels by S-lactoyl conjugates. Consistent with previous work in *E. coli*, we demonstrate that cytoplasmic acidification is the major glyoxalase dependent mechanism of MG protection in *B. subtilis.* The intracellular concentration of MG in *B. subtilis* can reach levels

as high as 0.3 mM (Landmann *et al.*, 2011), while BSH concentrations range from 0.3–3 mM during growth (Fig. 3). Since BSH is required for multiple cellular functions, the BSH pool may be substantially reduced under conditions of maximal MG production, leaving the cell vulnerable to additional insults. Thus, the cell may benefit from a rapid mechanism to protect itself from MG while maintaining a sufficient BSH pool. Consistent with models developed for MG resistance in *E. coli* (Ozyamak *et al.*, 2010), we therefore suggest that cytoplasmic acidification through activation of K^+ channels offers a more rapid means of protection than can be provided by the enzymatic conversion of MG to D-lactate by glyoxalase I and II. Ultimately, the combined actions of glyoxalase I and II serve to replenish the BSH pool, although the results in Fig. 3 suggest that this may be a relatively slow process. The mechanism of protection conferred by cytoplasmic acidification is poorly understood. However, it is suggested that the main role of cytoplasmic acidification is to protect cells from MG-induced DNA damage (Ferguson *et al*., 2000).

Determination of BSH-independent MG detoxification pathways

Next, we investigated candidate BSH-independent pathways of MG resistance in *B. subtilis*. We first searched for a *B. subtilis* homolog of glyoxalase III (HchA in *E. coli*), which converts MG directly to D-lactate (Subedi *et al.*, 2011). Using the Phyre2 structural homology search program (Kelley and Sternberg, 2009), we identified YdeA as a possible structural homolog of *E. coli* glyoxalase III, a DJ-1/PfpI family protein. In addition, a previous study showed that the transcription of two additional DJ-1/PfpI family proteins, YraA and YfkM, is increased upon MG exposure and that a *yraA yfkM* double mutant is more sensitive to MG than wild-type (Nguyen *et al.*, 2009). Indeed, consistent with previous results, *yraA* and *yfkM* single mutants are no more sensitive to MG than wild-type, whereas a *yraA yfkM* double mutant is more sensitive. A *ydeA* mutant was slightly more sensitive to MG than wild-type, which suggests that YdeA is also involved in MG detoxification in *B. subtilis* (Fig. 6A). *ydeA bshC* and *yraA yfkM bshC* mutants displayed increased sensitivity when compared to the single mutants, consistent with the notion that YdeA, YraA, and YfkM represent BSH-independent MG detoxification enzymes orthologous to *E. coli* glyoxalase III.

Thiol-independent MG detoxification in *E. coli* can also be carried out by broad specificity aldo-keto reductases, which comprise a large superfamily of NADPH-dependent enzymes that can reduce aldehydes and ketones (Ko *et al.*, 2005). An interesting candidate for this function in *B. subtilis* is AdhA, an aldehyde dehydrogenase whose expression is induced by MG (Nguyen *et al.*, 2009). However, an *adhA* mutant does not have an increased sensitivity to MG (V. Patel and JDH, unpublished data).

A second candidate for a BSH-independent detoxification mechanism is the YhdN broad specificity aldo-keto reductase that has been shown previously to reduce MG to acetol *in vitro* (Ehrensberger and Wilson, 2004). The *yhdN* gene is a member of the large σ^B-dependent stress and stationary phase regulon (Petersohn *et al.*, 1999). Expression of the σ^B regulon is induced under various stress conditions including exposure to heat, ethanol, high salt, starvation, or oxidative stress. Thus, expression of YhdN may occur in response to elevated levels of oxidative protein damage caused by MG. A *yhdN* null mutant has increased sensitivity to MG compared to wild-type and is even more sensitive than a BSH null strain (Fig. 6B). This suggests that YhdN is involved in MG resistance *in vivo* and may contribute to a larger degree than the BSH-dependent pathway under these conditions.

In *E. coli*, four different broad specificity aldo-keto reductases (AKRs), YafB, YqhE, YeaE and YghZ, have been implicated in MG detoxification, but their aggregate contribution to MG resistance has not been assessed (Ko *et al.*, 2005). We tested the contribution of five additional putative *B. subtilis* aldo-keto reductases (YvgN, YccK YqkF, YrpG, and IolS) to

MG resistance. None of corresponding null mutations (alone or in combination with a *yhdN* null mutation) increased MG sensitivity, suggesting that YhdN is the major aldo-keto reductase responsible for MG detoxification. Structural studies of the AKRs suggest that the substrate specificity is a result of differences in the active site architecture of each enzyme (Ehrensberger and Wilson, 2004; Marquardt *et al.*, 2005). Interestingly, YvgN has glyoxal/methylglyoxal reductase activity *in vitro* (Sakai *et al.*, 2001), yet deletion of *yvgN* has no effect on MG sensitivity. This may be explained, in part, by the lower catalytic efficiency of YvgN (k_{cat}/K_M of 1.58 s^{-1} mM^{-1}) (Lei *et al.*, 2009) when compared to YhdN (k_{cat}/K_M of 7.20 s^{-1} mM^{-1}) (Ehrensberger and Wilson, 2004). Our results indicate that, in *B. subtilis*, the *yhdN bshC* double mutant was more sensitive to MG than either single mutant, and the *yhdN ydeA bshC* triple mutant was more sensitive than the *yhdN bshC* double mutant, consistent with a model in which BSH-dependent activation of the KhtU transporter, the YhdN aldo-keto reductase, and the GlxC enyzmes all contribute to distinct MG detoxification pathways (Fig. 1).

Conclusions

This study provides insight into the role of the low molecular weight thiol BSH in MG resistance in *B. subtilis* and additionally defines two BSH-independent pathways of MG detoxification. The BSH-dependent glyoxalase I/II system (GlxA and GlxB) confers protection against MG primarily through cytoplasmic acidification resulting from the activation of the KhtSTU K^+ efflux pump and secondarily by converting MG to D-lactate. We also identify three proteins (YdeA, YfkM, and YraA) as candidate glyoxalase III enzymes, capable of converting MG to D-lactate in a BSH-independent manner, and demonstrate genetically that all three contribute to survival in cells challenged with exogenous MG. The third means of MG detoxification relies on the YhdN aldo-keto reductase, which converts MG to acetol. This is the single most important pathway, as judged by the level of sensitivity to MG, under conditions of excess MG in the environment. The relative contributions of these three pathways to MG produced as a natural byproduct of metabolism awaits further study. We do not exclude the possibility that there may be additional MG detoxification pathways in *B. subtilis*, perhaps involving alcohol dehydrogenases or aldehyde reductase which play a minor role in MG resistance in *E. coli* (Misra *et al.*, 1996). While there are many similarities between the *E. coli* and *B. subtilis* detoxification systems, the nature of the precise interactions between BSH and BSH-interacting proteins will be of interest given the differences between the structures of GSH and BSH.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Table 1. Bacteria were grown in the media described in the following sections. When necessary, antibiotics were used at the following concentrations: chloramphenicol (10 μg ml^{-1}), kanamycin (15 μg ml^{-1}), spectinomycin (100 μg ml^{-1}), and tetracycline (5 μg ml^{-1}). Gene deletions were constructed using long flanking homology PCR as previously described. Chromosomal DNA transformations were performed as described (Harwood and Cutting, 1990).

Disk diffusion assays

Strains were grown in LB at 37°C with vigorous shaking to an OD_{600} ~0.4. A 100 μl aliquot of these cultures was added to 4 ml of LB soft agar (0.7% w/v agar) and poured on to prewarmed LB agar plates. The plates were then allowed to solidify for 10 minutes at room temperature in a laminar flow hood. Filter disks (0.6 mm) were placed on top of the agar and

methylglyoxal (27.5 mmol) was added to the disks and allowed to absorb for 10 minutes. The plates were then incubated at 37C for 16–18 hours. The diameter of the zone of inhibition was measured.

Thiol quantification

For thiol analysis, the frozen cell pellets were derivatized with monobromobimane (mBBr) as previously described (Newton *et al.*, 1981), with some alterations. Briefly, 20 µl of bimane mix (50% acetonitrile, 2mM mBBr, 20 mM HEPES pH 8.0) were added per milligram of estimated residual dry weight. The samples were incubated at 60 °C for 10 minutes in the dark, and then cooled on ice. To acidify the sample, 5 M methanesulfonic acid was added and the cell debris was removed by centrifugation. The cell pellet was dried in an oven at 60° C overnight and weighed the next day. The supernatant was subsequently filtered through a 0.22 µm membrane and diluted in 10 mM methanesulfonic acid for thiol quantification by analytical HPLC. HPLC was performed with a HiChrom ACE-AR C18 4.6×250 mm, 5 µm, 100Å column equilibrated at 37°C with 99% Solvent A (0.25% v/v acetic acid and 10% methanol, adjusted to pH 4 with NaOH) 1% Solvent B (90% methanol). Samples were eluted with a gradient of Solvent B with a 1.2 ml min^{-1} flow rate: 0–5 min, 1% B; 5–15 min, 0–20% B; and 15–20 min, 20–100% B, followed by re-equilibration and re-injection. The total acquisition running time was 32 minutes. Detection was carried out with 10x gain with a Jasco fluorescence detector with excitation at 385 nm and emission at 460 nm. The thiol quantification was based on the standard fluorescence at each run. Five hundred pmol of mBBr-labelled bacillithiol and cysteine standards were injected as quantified controls. Concentrations of BSH, expressed as µmol per gram dry weight, can be converted to an estimate of cytosolic concentrations by dividing by 3 as previously published (Newton et al., 2009).

Cytoplasmic pH measurements

Measurement of cytoplasmic pH was based on the method described previously (Kitko *et al.*, 2009; Martinez *et al.*, 2012). Strains containing pMMB1311, which encodes GFPmut3, were grown for 16–18 hours in modified M63 media (0.745 g l^{-1} KCl, 2 g l^{-1} casein hydrolysate, 2 g l^{-1} ($NH_{42}SO_4$, 0.4 g l^{-1} KH_2PO_4 and 0.4 g l^{-1} K_2HPO_4) buffered at pH 7 with (50 mM MOPS) to decrease background fluorescence with 5 µg ml^{-1} tetracycline. The cultures were then diluted in fresh M63 media and grown at 30°C with aeration to an OD_{600} ~0.4. Cells were then harvested by centrifugation, resuspended to an OD_{600} ~0.4 in modified M63 medium, and 3 ml of culture was transferred to a quartz cuvette (2 mm path length). Aeration was provided by stirring. GFPmut3 excitation was measured from 480 to 510 nm (slit width, 10 nm) and an emission wavelength of 545 nm (slit width 20 nm) using a Perkin Elmer LS55 Luminescence Spectrometer. For experiments in which MG was used, 1.5 mM MG was added to the cuvette at time 0.

Standard curves correlating internal pH with fluorescence intensity were generated by obtaining fluorescence measurements of cells resuspended at in 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffered at pH values of 6.3, 6.8, 7.4, and 7.7. The cytoplasmic pH was equilibrated with the external pH by addition of 10 µM nigericin, an ionophore that collapsed the pH across the membrane. An equation was fit to the signal intensity curve and used to extrapolate pH values from fluorescence measurements.

MG survival assays

Strains were grown in modified M63 media buffered with 50 mM MOPS pH 7 at 37 °C to an OD_{600} ~0.4. At time zero, MG was added to the culture at to a final concentration of 3 mM. At various time points after MG addition, aliquots of the culture were harvested by centrifugation, then washed and diluted with fresh M63 media. For experiments in which

sodium benzoate was used, 30 mM sodium benzoate was added 5 min after MG addition. The cells were then spread on to LB plates and allowed to incubate for 16–18 hours at 37°C. Colonies were then counted and the percent survival was calculated as the number of colonies present with the addition of 3 mM MG divided by the number of colonies present without MG addition.

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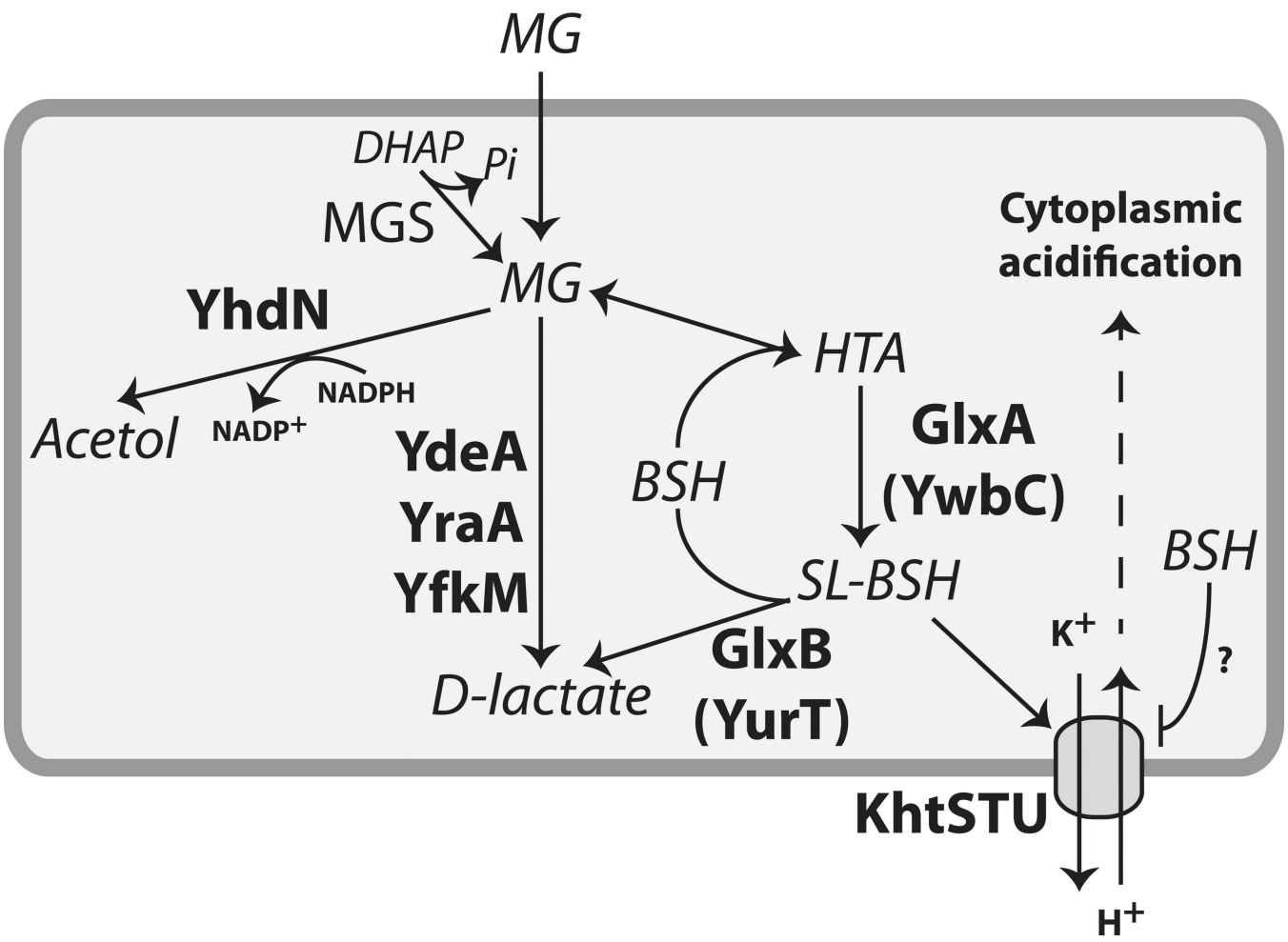

Figure 1. Summary of BSH-dependent and BSH-independent MG detoxification pathways

Proteins demonstrated as important for MG detoxification *in vivo* by this study are highlighted in bold. For proteins that have been renamed, the previous name is given below the new name in parentheses.

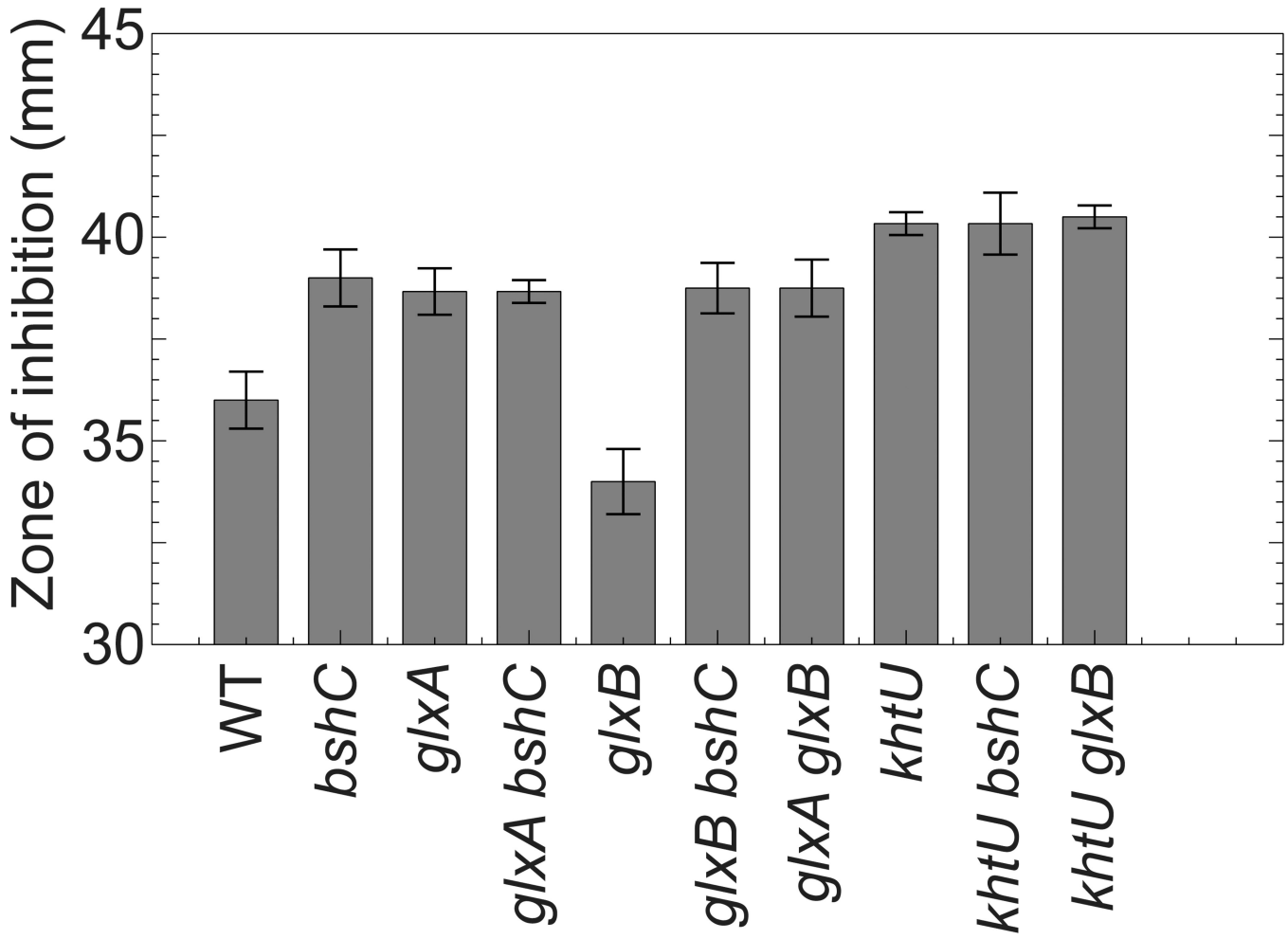

Figure 2. Determination of BSH-dependent MG detoxification pathway

Susceptibility of wild-type and mutant strains to MG (27.5 mmol) was tested by disk diffusion assay. The zone of inhibition is expressed as the diameter of the clearance zone in millimeters. The mean and standard deviation from at least three biological replicates is shown.

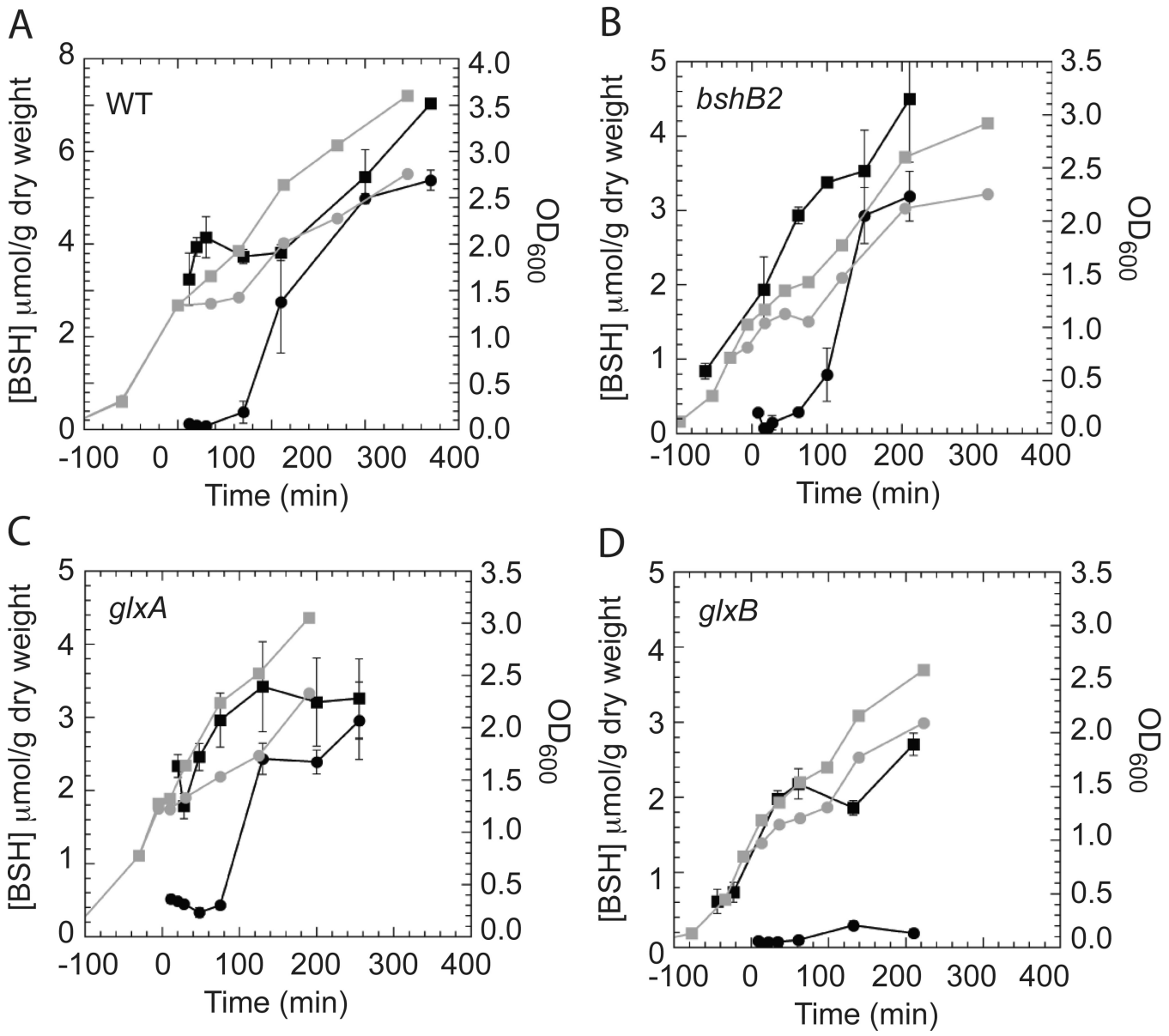

Figure 3. Recovery of BSH after MG challenge requires GlxB

Cellular BSH concentration as a function of time after MG addition in wild-type (A), *bshB2* (B), *glxA*(C) and *glxB* (D) mutant strains. Cells were challenged with 1 mM MG (time 0) and harvested at the indicated time points. Representative growth curves are shown for cells grown in the absence (grey squares) and presence (grey circles) of MG. Cellular BSH levels in the absence (black squares) and presence (black circles) of MG were quantified by HPLC after derivatization with monobromobimane. The average and standard deviation calculated from three independent experiments are shown.

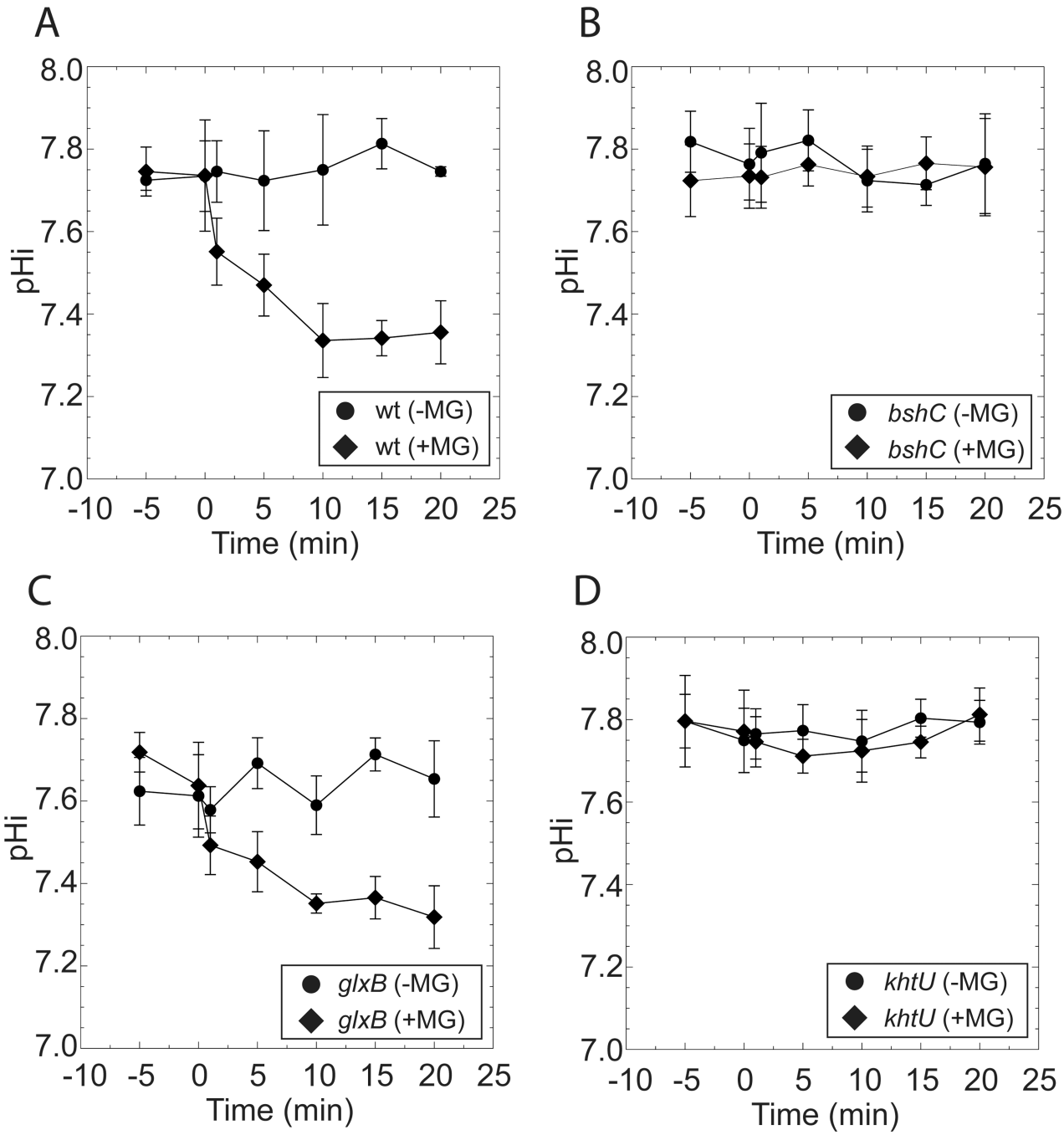

Figure 4. MG exposure leads to BSH and KhaSTU-dependent cytoplasmic acidification

Intracellular pH values for wild-type (A), *bshC* (B), *glxB* (C), and *khtU* (D) strains in the presence of MG. 1.5 mM MG was added at time 0. The averages and standard deviation calculated from three independent experiments are shown.

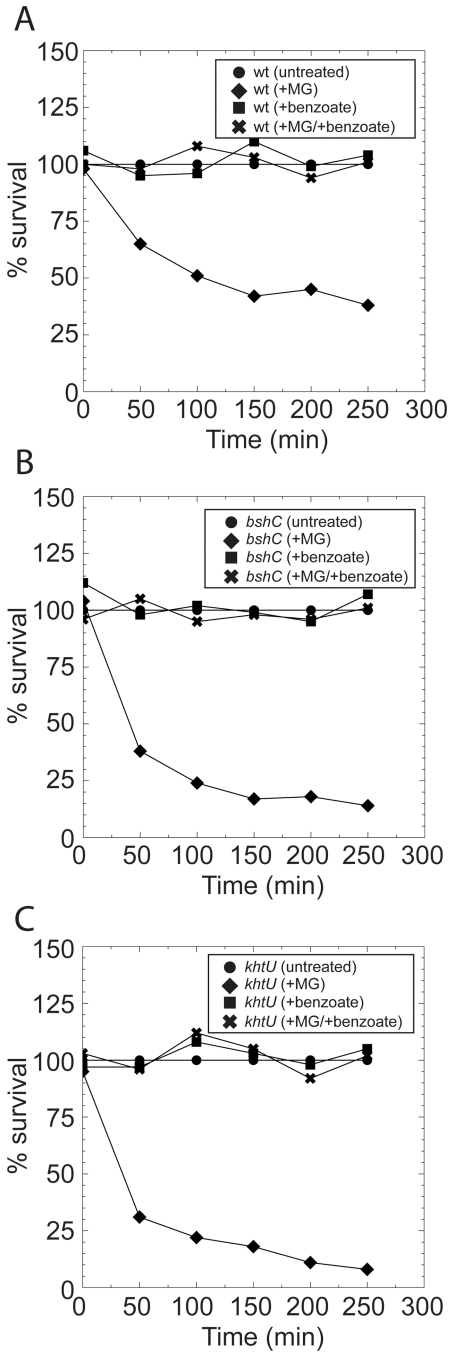

Figure 5. Cytoplasmic acidification is sufficient for protection from MG exposure

Wild-type (A), *bshC* (B), and *khaU* (C) strains grown in modified M63 media buffered to pH 7.0 with 50 mM MOPS were challenged at time 0 with 3 mM MG. After 5 minutes of MG treatment, 30 mM of sodium benzoate was added. Aliquots were removed at various times after MG addition, then diluted, spread on LB plates, and incubated at 37°C overnight. Percent survival was calculated as the number of colonies that grew after treatment divided by the number of colonies that grew in the absence of treatment multiplied by 100.

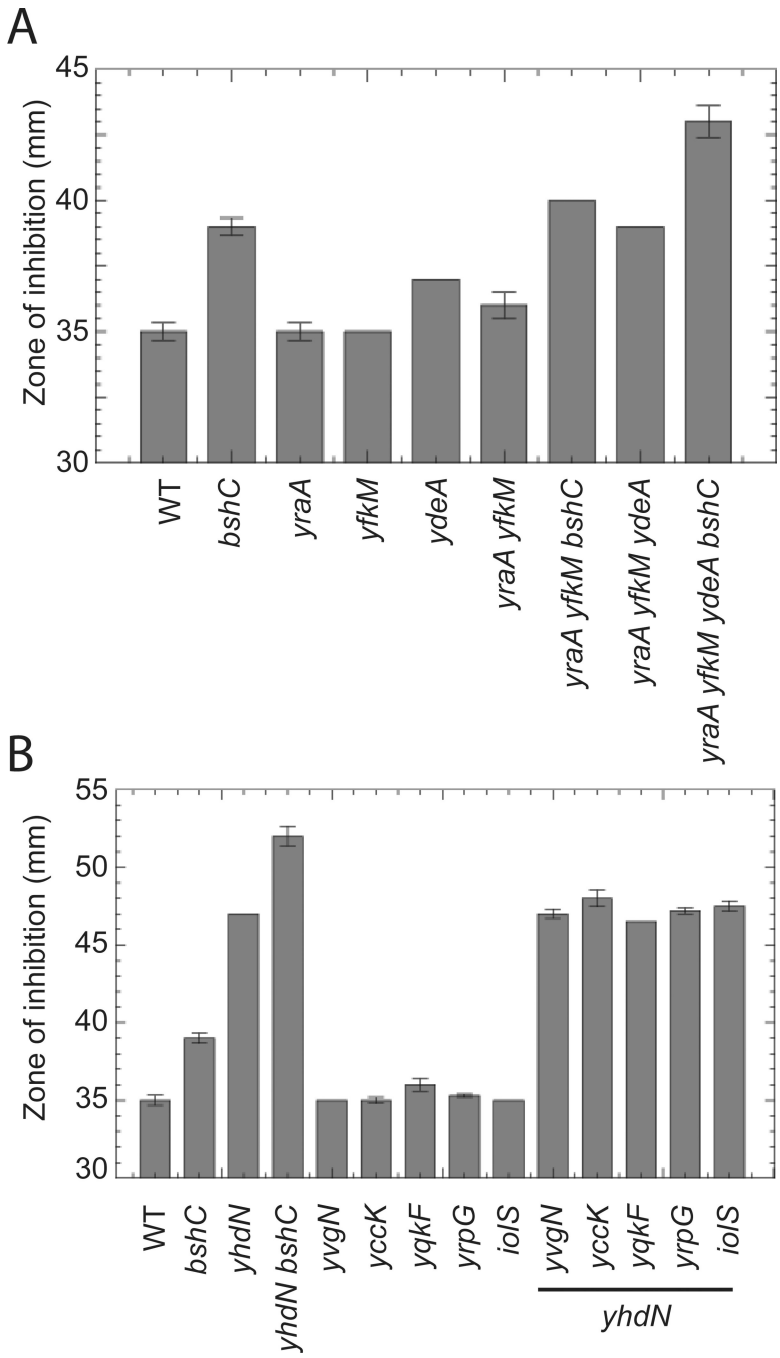

Figure 6. Contribution BSH independent pathways to MG resistance

Susceptibility of (A) putative glyoxalase III and (B) aldo-keto reductase mutant strains to MG (27.5 mmol) was tested by disk diffusion assay. The zone of inhibition is expressed as the diameter of the clearance zone in millimeters. The mean and standard deviation from at least three biological replicates is shown.

Table 1

Strains used in this study

Strain	Genotype	Plasmid	Source
CU1065	W168 *att* SPβ *trpC2* (wild type)		Laboratory stock
HB11042	CU1065 *bshB2::spec*		Gaballa *et al*., 2010
HB11069	CU1065 *glxB::kan*		This work
HB11079	CU1065 *bshC::kan*		Gaballa et al., 2010
HB11212	CU1065 *bshC::mls*		This work
HB16505	CU1065 *glxA::cm*		This work
HB16506	CU1065 *khtU::tet*		This work
HB16507	CU1065 *glxA::cm bshC::kan*		This work
HB16513	CU1065 *glxC::cm*		This work
HB16515	CU1065 *glxC::cm bshC::kan*		This work
HB16520	CU1065 *yhdN::kan*		This work
HB16533	CU1065 *yhdN::kan bshC::mls*		This work
HB16544	CU1065 *glxB::kan bshC::mls*		This work
HB16545	CU1065 *glxA::cm glxB::kan*		This work
HB16546	CU1065 *khtU::tet glxB::kan*		This work
HB16547	CU1065	pMMB1309	This work
HB16548	CU1065 *bshC::kan*	pMMB1309	This work
HB16549	CU1065 *glxB::kan*	pMMB1309	This work
HB16550	CU1065 *khtU::tet*	pMMB1309	This work
HB16551	CU1065 *yhdN::kan glxC::cm bshC::mls*		This work
HB16571	CU1065 *yraA::tet*		This work
HB16572	CU1065 *yfkM::spec*		This work
HB16573	CU1065 *yraA::tet yfkM::spec bshC::kan*		This work
HB16574	CU1065 *yraA::tet yfkM::spec ydeA::cm* CU1065 *yraA::tet yfkM::spec ydeA::cm*		This work
HB16575	*bshC::kan*		This work
HB16576	CU1065 *yvgN::cm*		This work
HB16577	CU1065 *yccK::tet*		This work
HB16578	CU1065 *yqkF::cm*		This work
HB16579	CU1065 *yrpG::mls*		This work
HB16580	CU1065 *iolS::spec*		This work
HB16581	CU1065 *yvgN::cm yhdN::kan*		This work
HB16582	CU1065 *yccK::tet yhdN::kan*		This work
HB16583	CU1065 *yqkF::cm yhdN::kan*		This work
HB16584	CU1065 *yrpG::mls yhdN::kan*		This work
HB16585	CU1065 *iolS::spec yhdN::kan*		This work