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Forum Review

The Many Faces of Glutathione in Bacteria

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ABSTRACT

Glutathione is one of the most abundant thiols present in cyanobacteria and proteobacteria, and in all mitochondria or chloroplast-bearing eukaryotes. In bacteria, in addition to its key role in maintaining the proper oxidation state of protein thiols, glutathione also serves a key function in protecting the cell from the action of low pH, chlorine compounds, and oxidative and osmotic stresses. Moreover, glutathione has emerged as a posttranslational regulator of protein function under conditions of oxidative stress, by the direct modification of proteins via glutathionylation. This review summarizes the biosynthesis and function of glutathione in bacteria from physiological and biotechnological standpoints. *Antioxid. Redox Signal.* 8, 753–762.

INTRODUCTION

GLUTATHIONE (GSH), γ-L-glutamyl-L-cysteinyl-glycine, is the most abundant nonprotein thiol found in many organisms (17, 18, 57). The physiological concentration of GSH ranges from 0.1 to about 10 m*M* in bacteria (17, 18, 48). Because of its two carboxyls, one amine, and one thiol group, GSH is highly soluble in aqueous solutions and in polar solvents. The thiol group in GSH is responsible for its biological activity, whereas the gamma linkage between the glutamic acid and cysteine prevents its degradation by proteases. Only one kind of peptidase, γ-glutamyl transpeptidase (GGT), is known to hydrolyze GSH by cleaving the gamma linkage between glutamate and cysteine and to transfer the glutamyl residue to another amino acid. At a pH of 7.0 and 25°C the redox couple formed between glutathione and its disulfide-bonded dimeric form, GSSG, exhibits a reduction potential ($E^{\circ\prime}$) of −240 mV (63). The ratio of GSH:GSSG in the cytoplasm is carefully controlled and GSH is kept mostly in its reduced state. In *Escherichia coli* the GSH:GSSG ratio has been estimated to be around 200 for cells growing in LB medium (2), which corresponds to a redox potential of −240 mV, assuming a total intracellular glutathione concentration of 5 m*M*, pH 7.0, and 25°C.

In the cytoplasm GSH serves as a protein reductant, either directly or through the reduction of the glutaredoxin system of enzymes. The principal, albeit possibly not the only, mechanism for the reduction of oxidized GSH is glutathione reductase (the product of the *gor* gene) which uses reducing equivalents from NADPH. Glutathione reductase thus serves as the key link between the two redox couples (GSH/GSSG and NAD(P)H/NAD(P)) in the cell. Because the glutathione and NAD(P)H/NAD(P) systems do not exchange electrons directly at any appreciable rate, the two redox couples can be maintained within the cell at different redox potentials as required for a variety of cellular functions.

GSH is present in almost all eukaryotes with the exception of those that do not have mitochondria or chloroplasts, but its production among prokaryotes is restricted to cyanobacteria and proteobacteria, as well as a few strains of gram-positive bacteria (18, 53). No glutathione has been found in any of the other subgroups of eubacteria or in archaebacteria with the exception of the green sulfur eubacteria where glutathione is present at very low concentrations (in the micromolar range). Some of the prokaryotes that lack glutathione seem to produce different low molecular weight thiols which appear to function in a similar way to GSH. For instance, anaerobic sulfur bacteria use glutathione amide, whereas the major thiols in aerobic phototrophic halobacteria and in actinomycetes are γ-glutamylcysteine and mycothiol, respectively (16, 53). While not essential in *E. coli*, GSH plays a critical role in protection against environmental stresses that include osmotic

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shock, acidity, protection against toxins like methylglyoxal, chlorine compounds like hypochlorous acid and monochloroamine, and oxidative stress induced by peroxides, such as hydrogen peroxide (H_2O_2) or alkyl hydroperoxides (Fig. 1). GSH is also involved in the regulation of intracellular potassium levels and in preventing the formation of aberrant protein disulfides in the cytoplasm.

BIOSYNTHESIS AND DEGRADATION OF GLUTATHIONE

Glutathione is synthesized in two steps:

$$\text{L-Glu} + \text{L-Cys} + \text{ATP} \leftrightarrow \gamma\text{-Glu-Cys} + \text{ADP} + P_i \quad \text{(i)}$$

$$\gamma\text{-Glu-Cys} + \text{Gly} + \text{ATP} \leftrightarrow \text{GSH} + \text{ADP} + P_i \quad \text{(ii)}$$

In *E. coli*, reactions (i) and (ii) above are catalyzed by the products of the *gshA* and *gshB* genes, respectively. The *gshA* gene encodes the cytosolic ATP-dependent enzyme γ-glutamylcysteine synthetase (GCS) that catalyzes the addition of glutamic acid to cysteine to form γ-glutamylcysteine [reaction (i)]. The *E. coli* GCS is a monomer of 58.3 kDa (31) as opposed to the well-characterized rat kidney GCS that is a heterodimer (60, 64). The *E. coli* GCS crystal structure has been solved recently (28). Both the bacterial and the eukaryotic enzymes are feedback inhibited by glutathione, which binds to the glutamate binding site on the active site and at another position that interacts with the thiol group of GSH (31). The product of the *gshB* gene is the cytosolic ATP-dependent enzyme, glutathione synthetase (GS), which is a tetramer with four identical subunits of 35.6 kDa (83) that catalyzes the addition of glycine to γ-glutamylcysteine to form GSH. Some gram-positive bacteria, such as enterococci, streptococci, and *Listeria monocytogenes*, surprisingly produce significant amounts of GSH, despite the apparent lack of a *gshB* gene (24). However, an ORF in the genome of *L. monocytogenes* had been predicted to contain an N-terminal domain that encodes a molecule similar to bacterial γ-glutamylcysteine synthetases and a C-terminal domain that encodes a molecule with some resemblance to bacterial glutathione synthetases (11). This observation paved the way for the isolation of a multidomain fusion protein in *L. monocytogenes* that catalyzes both reactions for glutathione biosynthe-

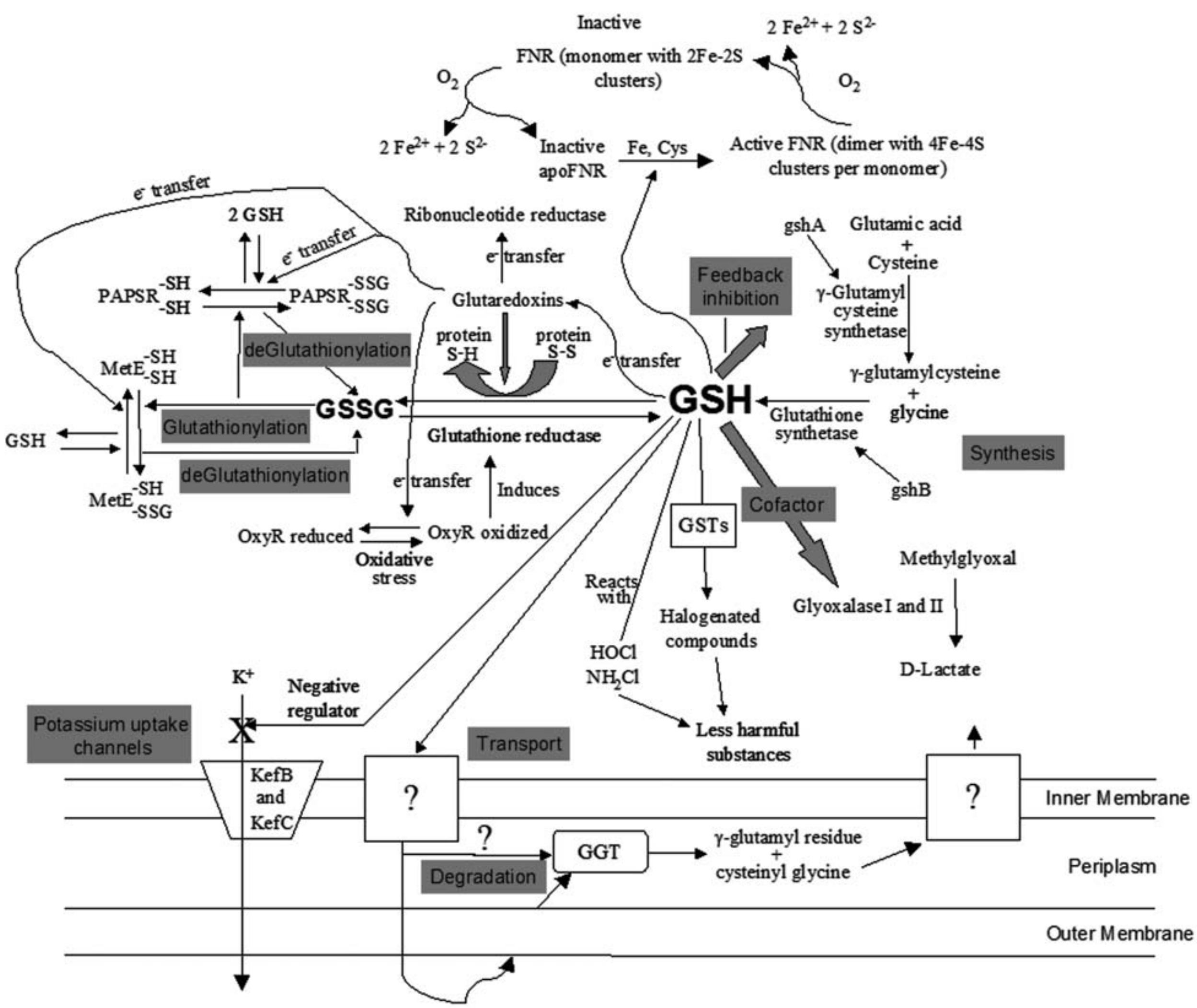

FIG. 1. The many faces of glutathione in *E. coli*.

sis in this organism (24). Around the time of this discovery, a similar fusion protein was isolated from *Streptococcus agalactiae* (32).

Because of its γ-linkage, GSH cannot be degraded easily and there is only one peptidase, γ-glutamyl transpeptidase, which is known to degrade GSH. GGTs are widely distributed among living organisms, from bacteria to mammals. Much more is known about mammalian GGTs, for which an extensive literature exists (10, 75), than their bacterial counterparts. In fact, only four nucleotide sequences of bacterial GGTs have been reported (74). In bacteria, GGT localization varies between different organisms; interestingly, in *E. coli* GGT is a soluble periplasmic protein (73), whereas in *Neisseria meningitidis* GGT seems to be associated with the inner membrane facing the cytoplasmic side (74). The *E. coli* GGT consists of a large subunit and small subunit that are generated from a single precursor through an autocatalytic post-translational modification (72). The *E. coli* GGT has been proposed to serve as a part of a pathway to use γ-glutamyl peptides as an amino acid source (70). It plays a role in breaking the gamma linkage in GSH and, as a consequence, is involved in catalyzing the first step of the salvage of cysteine (71).

ROLES OF GLUTATHIONE IN BACTERIA

Osmotic stress

In *E. coli* osmoadaptation to a higher osmolarity medium starts with the rapid accumulation of K^+ and the counter ion glutamate (12, 65) which promote turgor recovery. Because of the charged nature of the K^+-glutamate pair, the increase in their concentration during osmoadaptation can reach an upper level of approximately 400 m*M* in gram-negative bacteria. Beyond that point, this primary response has too many detrimental consequences to the cell, mostly because of the disturbance of the cytoplasmic ionic balance. Hence, to adapt to a higher osmolarity medium, cells initiate a secondary response that consists of accumulating neutral osmoprotectants such as glycine, betaine, carnitine, and proline that can reach very high concentrations without affecting cellular processes because of their lack of net charge (65). The concentration of GSH in *E. coli* has been found to increase during osmotic shock (12). An *E. coli* strain deficient in GSH synthesis (*gshA* mutant) was unable to grow in media with osmolarities above 1.4 os*M*, as opposed to the 2.0 os*M* limit for the wild-type bacteria. Also, the growth rate of the mutant was found to be lower in media with intermediate osmolarities (47). Even though GSH is involved in the regulation of K^+ export channels (49) (*gshA* mutants have higher rates of K^+ efflux), it has been shown that the effect of GSH on osmoadaptation is not due to a change in K^+ retention (47). At this point, the exact role of GSH during osmotic shock is not yet clearly understood but there is some evidence that it may be related to its function as an antioxidant. In particular, osmotic shock in *E. coli* is accompanied by reactions characteristic of oxidative stress, such as an increase in the induction of SoxS and SodA (67). Thus, GSH may be part of an oxidative stress response that is induced by osmotic shock and may play a role in maintaining cell viability in hyperosmotic environments (66).

Low pH

There is substantial evidence that GSH plays a role in protecting cells from exposure to acidic conditions. In *E. coli*, the potassium export channels KefB and KefC are inhibited by GSH, and in the absence of GSH, K^+ leaks out of the cell (49, 50). K+ efflux has been linked to a decrease in cytoplasmic pH (20) and, even though cytoplasmic pH homeostasis is not completely understood, it has been proposed to be dependent on Na^+ and K^+ transport (6, 7). Thus, the effect of GSH on low pH protection may be related to its involvement with K^+ regulation. Consistent with this hypothesis, it has been observed that in *E. coli* there is increased expression of Kdp, a high affinity potassium uptake system, under low pH conditions (1, 46) and that in a strain unable to produce GSH (*gshA* mutant) there is a decrease in the cytoplasmic pH in media with a low concentration of K^+ (20). Therefore, a possible reason for the sensitivity of *gshA* mutants to low pH may be due to the inability of the cells to maintain an optimal intracellular K^+. Supporting this explanation, a mutant of *Rhizobium tropici* (a member of the alphaproteobacteria) unable to produce GSH, due to a transposon insertion within a gene exhibiting high similarity to the *E. coli gshB* gene, is not capable of growing in acidic media at pH < 5 (59). Furthermore, the wild-type strain has a higher intracellular K^+ concentration than the GSH mutant under acidic conditions.

Protection from methylglyoxal

The methylglyoxal pathway, an energetically unfavorable bypass of glycolysis reactions, is thought to provide a mechanism for alleviating the stress caused by switching from low to elevated levels of sugar phosphates (21, 77). In bacteria methylglyoxal is synthesized mainly from the glycolytic intermediate, dihydroxyacetone phosphate, via the action of methylglyoxal synthase. This enzyme is feedback inhibited by inorganic phosphate (P_i) and is allosterically controlled by dihydroxyacetone phosphate (30). The allosteric effect of dihydroxyacetone phosphate on methylglyoxal synthase results in elimination of the feedback inhibition by P_i. Thus, elevated levels of methylglyoxal are produced when there is an accumulation of dihydroxyacetone phosphate because P_i inhibition is alleviated and methylglyoxal synthase overproduces methylglyoxal (19, 34). GSH plays a major role in the protection of *E. coli* cells against the toxicity of methylglyoxal (20). First of all, GSH is required in the first step of detoxification by the enzymes glyoxalase I and II that detoxify methylglyoxal to D-lactate via the formation of two metabolites, a hemithioacetal and *S*-lactoylglutathione. Second, as mentioned above, GSH is a negative regulator of the KefB and KefC K^+ efflux systems. Depletion of GSH by the glyoxalase I and II enzymes partially activates the KefB and KefC efflux systems but full activation requires the formation of glutathione adducts like *S*-lactoylglutathione. Third, the activation of KefB and KefC causes leakage of K^+ that results in an influx of protons into the cytoplasm leading to a decrease in intracellular pH (20), which in turn protects the cell from methylglyoxal toxicity. Consistent with this mechanism, conditions that increase the intracellular pH, or reduce the pH drop when the KefB and KefC K^+ efflux channels open, make *E. coli* more susceptible to methylglyoxal. However, the precise mechanism by which lower intracellular pH protects *E. coli* from methylglyoxal is not known. It has been proposed that a lower pH may protect the cell by reducing damage to DNA via the activation of DNA repair mechanisms or perhaps that low pH prevents the interaction of electrophiles, such as methylglyoxal, with cellular macromolecules (21).

Chlorine compounds

GSH protects *E. coli* from hypochlorous acid (HOCl) and monochloroamine (NH_2Cl) by reacting directly with these chlorine compounds to produce less harmful substances (9). Current evidence indicates that in bacteria this reaction is spontaneous and not mediated by enzymes such as glutathione *S*-transferases (GSTs) (9). In addition, it has been shown that HOCl elicits a response similar to H_2O_2 oxidative stress (13, 14), and so it is possible that GSH could have an indirect effect via its role in oxidative stress responses.

GSTs catalyze the nucleophilic conjugation of both xenobiotic and endogenous electrophiles with GSH (80, 81). Thus, they may play a role in detoxification of halogenated compounds. In fact, in some bacteria, like nitrate-respiring *Hyphomicrobium sp.* and aerobic gram-negative facultative methylotrophic bacteria, GSTs are a central element in the metabolism of chlorinated hydrocarbons such as dichloromethane (DCM), which are used as carbon sources (41).

Oxidative stress

Oxidative stress occurs when cells are exposed to elevated levels of reactive oxygen species such as H_2O_2, alkyl hydroperoxides, and hydroxyl radicals (8, 69). The adaptive response to oxidative stress is regulated by OxyR and SoxRS transcription factors, which induce the expression of antioxidant activities in response to stress due to H_2O_2 and superoxides, respectively. During oxidative stress, activation of OxyR (25) induces the expression of a number of genes including glutathione reductase and glutaredoxin 1 (*grxA*) (8). This suggests a possible role of GSH in protective action towards oxidative stress, although there does not seem to be a straightforward involvement of GSH in protecting against H_2O_2. For instance, exponentially growing *E. coli* that lack GSH (*gshA* mutant) have normal resistance to H_2O_2 (26), but when they reach stationary phase they are more susceptible to killing by H_2O_2 (9). *E. coli gor* mutants show diamide sensitivity similar to *gshA* mutants, are somewhat sensitive to paraquat and cumene hydroperoxide, and show increased H_2O_2 sensitivity in a catalase mutant background (3, 8). GSH also plays an indirect role in cells under peroxide stress by reducing oxidized OxyR by means of glutaredoxin 1. Thus, when the oxidative challenge has passed, OxyR is restored to its reduced and transcriptionally inactive state (2, 84). Finally, GSH is also involved in protection against the damage caused by radiation under aerobic conditions (27).

Reduction of ribonucleotides and other substrates

Ribonucleotide reductase (RNR) is the enzyme responsible for the conversion of ribonucleotides to the corresponding deoxyribonucleotides to provide the precursors needed for DNA synthesis (33, 39). During the RNR catalytic cycle, a disulfide bond is formed in the active site between the two cysteines that are used to reduce the ribonucleotide substrate. In *E. coli*, reduction of the class Ia RNR that is utilized under aerobic conditions can be performed by three members of the thioredoxin fold superfamily: thioredoxin 1 (TrxA), thioredoxin 2 (TrxC), and glutaredoxin 1 (GrxA) (61). These proteins are part of the thioredoxin and glutaredoxin pathways which use NADPH as a source of reducing equivalents that are transferred to the corresponding thioredoxins (TrxA, TrxC) or glutaredoxins (GrxA, GrxB, GrxC) by means of a reductase. Glutathione is required for the reduction of the glutaredoxins by glutathione reductase (79). Under aerobic conditions RNR is an essential enzyme and therefore *E. coli* needs at least one of the two reducing pathways (Fig. 2) for viability (55). For a more in-depth discussion about RNR, see a review by Gon and Beckwith in this forum issue.

Other proteins that are reduced by the glutaredoxin pathway include arsenate reductase (ArsC), which catalyzes the reduction of arsenate (AsO_4^{3-}) to arsenite (AsO_3^{3-}), and 3′-phosphoadenylsulfate (PAPS) reductase which is part of the sulfur assimilation pathway and is required for growth with sulfate (SO_4^{2-}) as only source of sulfur (61). GSH is also responsible for directly reducing fumarate nitrate reductase regulator (FNR), an oxygen sensor regulator, which is one of the major proteins for controlling the switch from aerobic to anaerobic metabolism (35, 78). FNR is inactive during aerobic condi-

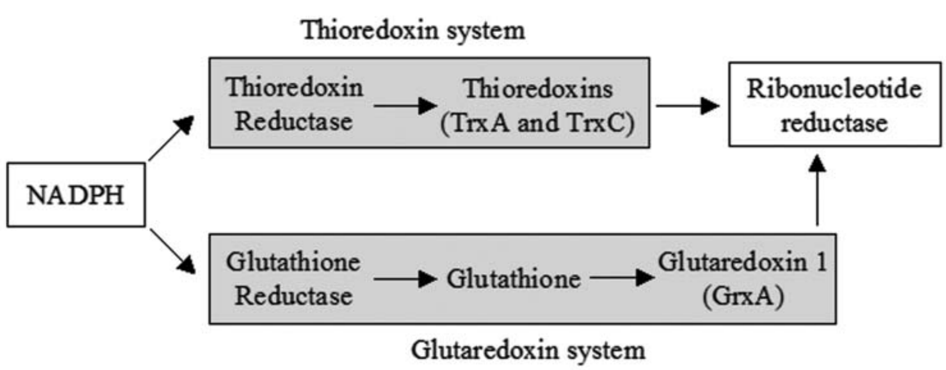

FIG. 2. Electron flow from NADPH to the essential enzyme ribonucleotide reductase in the thioredoxin and glutaredoxin systems. *Arrows* indicate the direction of electron flow.

tions as a result of the conversion of [4Fe–4S] cluster of the protein into a [2Fe–2S] cluster and further to apoFNR which lacks the Fe–S clusters. The reversion of FNR to the active state during anaerobic conditions requires GSH. The active FNR, a homodimer with one [4Fe–4S] cluster per subunit, binds to specific target DNA sites and controls the expression of genes responsible for growth under anaerobic conditions. Finally, for the sake of completion, it should be noted that methionine sulfoxide reductase, an essential enzyme when *E. coli* is grown in minimal media, is reduced by the thioredoxin pathway but it can also be reduced by the glutaredoxin pathway when glutaredoxin 1 is overexpressed (61).

Glutathionylation

Protein glutathionylation involves the formation of mixed disulfides between protein cysteines and glutathione. It has been estimated that in *E. coli* approximately 2% of the total glutathione content is in the form of mixed disulfides with proteins (51). Glutathionylation can occur either by direct oxidation of either protein thiol or GSH followed by formation of a mixed disulfide or alternatively, by thiol–disulfide exchange between oxidized glutathione (GSSG) and a protein cysteine. Current evidence seems to favor thiol oxidation as the predominant *in vivo* mechanism by which proteins are glutathionylated (29, 76). Protein glutathionylation is mostly found in cells under oxidative stress and could serve as a mechanism for the protection of reactive protein thiols from overoxidation to sulfinic acid or to higher oxidation states. Protein cysteines that get oxidized to sulfinic acid (Cys-SO_2H) cannot be reduced by normal cellular reductants, such as the glutaredoxin or thioredoxin pathways, thus resulting in their irreversible inactivation. It should be pointed out that in some eukaryotic cells it has recently been shown that this process is not always irreversible as previously thought (23). The formation of a mixed disulfide with GSH might serve to prevent the irreversible oxidation of protein cysteines so that they can be reduced to their native state when the organism has been removed from the oxidative environment (29). In fact, glutaredoxins are very efficient in the reduction of the mixed disulfides in glutathionylated proteins (61). Interestingly, glutaredoxin 2 (GrxB) is highly upregulated during stationary phase, reaching levels of up to 1% of total cell protein, but the exact physiological motivation for this increase remains to be elucidated (58).

While glutathionylation of proteins is thought to be a common mode of redox regulation in eukaryotes (38), very few proteins in *E. coli* are known to be regulated in this manner. In particular, glutathionylation has been shown to modulate the function of only three proteins: methionine synthase (MetE) (29), PAPS reductase (44), and possibly OxyR; although the occurrence of the glutathione adduct of OxyR *in vivo* is controversial (25). Very recently, it was shown that the *E. coli* glutaredoxin 4, a monocysteine glutaredoxin, can be reduced by thioredoxin reductase (TrxB) *in vitro* when glutathionylated (22). This is surprising because *E. coli* TrxB has a very narrow range of substrate specificity and is not capable of reducing oxidized glutaredoxins GrxA, GrxB or GrxC. It remains to be seen whether this finding has any regulatory implications *in vivo*.

E. coli cells under oxidative stress develop a methionine auxotrophy due to inactivation of MetE, the enzyme that catalyzes the final step of methionine biosynthesis. Experiments carried out by Hondorp *et al*. (29) demonstrated that the reason for MetE inactivation under oxidative stress is the glutathionylation of Cys-645 which seems to block access to the active site of the enzyme. Remarkably, *in vivo* and *in vitro* data indicate that the glutathionylation of MetE is reversible and may serve as a mechanism to protect the enzyme from irreversible oxidative damage.

PAPS reductase is a homodimeric enzyme that is responsible for reducing 3′-phosphoadenylsulfate during the reduction of inorganic sulfate to sulfite. PAPS reductase is required for growth when sulfate is the only sulfur source. During oxidative stress the enzyme is inactivated by glutathionylation of Cys-239 by a thiol–disulfide exchange mechanism (44). It has been proposed that inactivation by glutathionylation is a reversible mechanism for shutting down PAPS reductase activity to conserve reducing equivalents under oxidative stress conditions (44).

OxyR is a bacterial transcriptional activator induced during peroxide oxidative stress. Some controversy exists regarding the exact mechanism by which the tetrameric OxyR becomes activated (25). The most likely mechanism involves the formation of a disulfide between Cys-199 and Cys-208 (2, 84). Alternatively, activation may require modification of Cys-199 either by peroxide to form sulfenic acid, by reactive nitrogen species to form Cys199-SNO, or by oxidized glutathione resulting in glutathionylation of the protein (37). Even though only the latter process directly involves glutathionylation, it is important to point out that the GSH:GSSG ratio can also influence the formation of the Cys-199 and Cys-206 disulfide and thus play a role in OxyR activation (2).

Besides protein glutathionylation, glutathione also becomes conjugated to spermidine (*N*-(3-aminopropyl)-1,4-diaminobutane) in stationary phase (5), but the reason for the formation of this adduct is still unclear.

GLUTATHIONE HOMEOSTASIS

Under normal physiological conditions, the rate limiting step in the synthesis of glutathione is not the final step catalyzed by glutathione synthetase (GS) but the formation of γ-glutamylcysteine catalyzed by γ-glutamylcysteine synthetase (GCS) which, as mentioned before, is feedback inhibited by GSH (31, 52). In addition, cells have at least two more ways to control GSH production: via the availability of the amino acid precursors and through the regulation of *gshA*. The concentration of cysteine is limiting relative to glutamate. Thus, the intracellular pool of cysteine can exert an effect on the rate of GSH synthesis (45). The level of *gshA* expression does not seem to play a major role because the enzyme is feedback inhibited. For example, heterologous expression of *E. coli gshA* in *Saccharomyces cerevisiae* resulted in nearly 1000-fold higher enzyme level but the increase in the concentration of glutathione was only 2-fold (54). Thus, feedback inhibition and cysteine concentration are the two factors by which the cell primarily controls GSH production. It seems that under normal physiological conditions feedback inhibition is responsible for ensuring a constant level of GSH in the cell cytoplasm. Another means by which the cell can control the level of GSH is through its degradation by γ-glutamyl transpeptidases. However, in *E. coli*, GGT is a periplasmic protein and therefore it is unlikely to play a role in modulating the cytoplasmic GSH level. It is not clear whether efflux of GSH into the periplasm and degradation by GGT plays a role in homeostasis in the cytoplasm under physiological conditions. In fact, the presence of endogenous GSH in the periplasm has not been ascertained. An earlier report suggested that *E. coli* excreted large amounts of GSH into the extracellular fluid (56), but the mechanism responsible for this phenomenon has not been investigated. Similarly, there is no information as to whether GSH normally occurs in the periplasmic space or what role it plays in that compartment.

The mechanisms responsible for GSH homeostasis in bacteria appear to be only partially understood. Studies in our laboratory have led to the surprising finding that *trxB*(-) *gor*(-) *ahpC** cells (strain FA113) have a significantly higher level of free thiols due to the accumulation of reduced GSH. *E. coli* FA113 promotes the formation of disulfide bonds in the cytoplasm because the *trxB* mutation results in the accumulation of TrxA and TrxC in their oxidized state that in turn promotes disulfide bond formation (68). A *trxB gor* double mutant needs an exogenous reductant such as DTT to be able to grow at reasonable rate, presumably because of the requirement to have at least one of the two reducing pathways to reduce essential enzymes such as ribonucelotide reductase. When a *trxB gor* mutant strain is grown without DTT fast growing colonies that accumulate suppressor mutations readily appear (4). The nature of the suppressor mutation in strain FA113 has been identified and consists of the expansion of a triplet repeat resulting in the addition of one amino acid in the alkyl hydroperoxide reductase (AhpC). While AhpC is a peroxidase, the mutant enzyme is presumed to exhibit glutathione reductase activity; although only indirect evidence exists for this activity and it has not been demonstrated biochemically (62). We have found that the flow cytometric probe monobromobimane (mBBr) (40) can be employed for the determination of GSH levels in bacteria in a manner analogous to its use in eukaryotic cells (15). mBBr is rapidly taken up by the cell and reacts with free thiols in GSH to form a fluorescent product that cannot diffuse across the cytoplasmic membrane. LC–MS revealed that the only major low molecular weight species that reacts in cell lysates is GSH. Accordingly, no peak corresponding to an mBBr conjugated product could be detected in *gshA*(−) cells. HPLC analysis further showed that the GSH–mBBr peak in cell lysates from exponentially growing FA113 cells was increased substantially compared to the parental strain DHB4, consistent with the flow cytometric data. Measurements of the thiol content of lysed cells with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) are also consistent with the results obtained with mBBr (data not shown). Figure 3 and Table 1 show a comparison of the free thiol levels as measured with mBBr in different strain backgrounds and upon overexpression of *gshA* from a plasmid. Consistent with previous observations, overexpression of *gshA* did not result in an increased amount of free thiols, due to the feedback inhibition of GCS by GSH. The presence of elevated concentrations of GSH in *E. coli* FA113 is surprising for two reasons: first, because it indicates the existence of a mechanism that overrides the feedback inhibition of GCS to result in increased accumulation of GSH in the cell, and second, because GSH is reduced despite the fact that FA113 lacks glutathione reductase. Interestingly, *in vitro* experiments with cell extracts from FA113 show that this strain is still capable of reducing oxidized glutathione (36). Furthermore, we have observed that in FA113 the level of GSH, as detected by flow cytometry, does not change between exponential and stationary phase cells. These observations indicate that an unknown mechanism involved in GSH homeostasis in exponential phase cultures is impaired in *E.coli* FA113. The availability of a single cell probe for the detection of GSH by flow cytometry enables the application of genetic techniques for the analysis of this phenomenon and other aspects of GSH metabolism. Studies along these lines are on-going in our laboratory.

INDUSTRIAL PRODUCTION OF GLUTATHIONE

Glutathione is extensively used in the pharmaceutical, food, and cosmetic industries (82). GSH can be produced either enzymatically or by fermentation. Currently, the major method for industrial production is fermentation using yeast (*Saccharomyces cerevisiae* or *Candida utilis*), because of lower costs compared to enzymatic production which requires the addition of expensive amino acid precursors (glutamic acid, cysteine, and glycine). Some work has also been done on the production of GSH in bacteria (43). For example, an *E. coli* strain selected for resistance to methylglyoxal resulted in

TABLE 1. MEAN FLUORESCENCE OF *E. COLI* CULTURES AT LATE EXPONENTIAL AND STATIONARY PHASES

	DHB4 (no probe)	*DHB4 gshA*	*DHB4*	*DHB4 pTrc99a-gshA*	*DHB4 trxB gor ahpC**
Exponential	1.5	4.8	7.7	6.7	18.4
Stationary	1.7	6.5	16.6	16.7	18.7

Mean fluorescence of 10000 *E. coli* events for cells grown at 37°C with shaking until late exponential phase ($OD_{600} \approx 1$) or stationary phase (grown for 14.5 h). See Fig. 3 text for a description of the conditions of the flow cytometric assay.

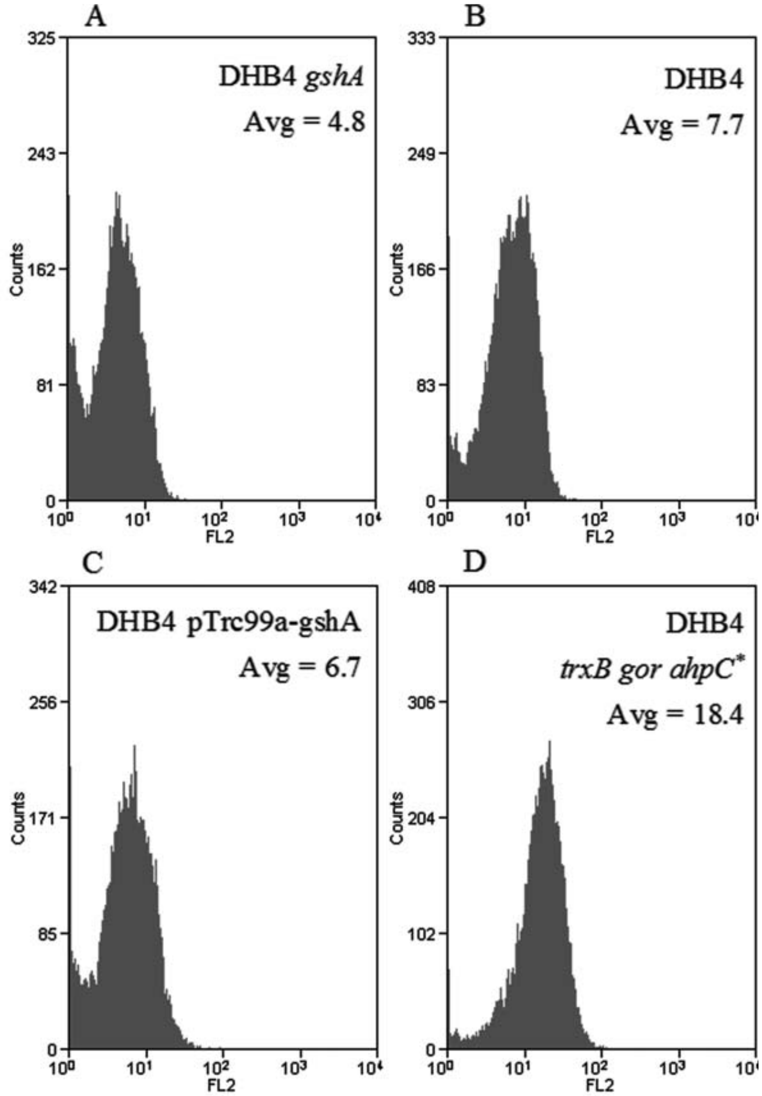

FIG. 3. Fluorescence distribution of *E. coli* in late exponential phase ($OD_{600} \approx 1$) following labeling with the thiol specific probe mBBr. Histograms represent the mean fluorescence of 10000 *E. coli* events. (**A**) WP758 (DHB4 *gshA*); (**B**) DHB4; (**C**) DHB4 pTrc99a–gshA; (**D**) FA113 (DHB4 *trxB gor ahpC**). *Flow cytometric assay conditions:* Cells from single colonies were grown overnight in $M9_{casein}$ media (1 X M9 minimal salts (Sigma, M6030), 0.4% (w/v) glucose, 0.1% (w/v) casein enzymatic hydrolysate (Sigma, C0626), 2 m*M* $MgSO_4$, 0.05 mg/ml thiamine) with the appropriate antibiotic at 37°C with shaking. Cells were then subcultured 1:100 in fresh $M9_{casein}$ media and grown to $OD_{600} \approx 1$ at which point 1 ml of cell culture was centrifuged (5 min at 10,000 RPM), the supernatant discarded and the pellet resuspended in 1 ml PBS. Cells were then incubated with monobromobimane (mBBr, Molecular Probes, M-1378) at a final concentration of 0.33 m*M* at room temperature for 60 min and analyzed for fluorescence emission with a flow cytometer (MoFLO, Cytomation). DHB4, WP758 and FA113 were obtained from Jon Beckwith's laboratory. Their genotype is as follows: DHB4 is MC1000 *phoA*(PvuII) *phoR malF3;* WP758 is DHB4 *gshA*::Tn10Kan; FA113 is DHB4 *gor522* . . . mini-Tn10Tc *trxB*::Kan *ahpC**.

1.6-fold higher levels of GSH when grown in the presence of methylgyoxal. Also, γ-glutamylcysteine synthetase variants that are desensitized for GSH feedback inhibition have been isolated and cloned, resulting in higher GSH levels (52). Recently the gram-positive bacteria *Lactococcus lactis* has been used as platform for GSH production. This bacterium has no endogenous GSH and shows no GGT activity. By transforming *L. lactis* with a plasmid with *E. coli gshA* and *gshB* genes, the strain achieved a GSH concentration of 140 m*M*, which is the highest concentration ever reported for a bacterial system (42). Surprisingly, GCS does not seem to be feedback inhibited in this strain although the reasons for that remain unclear.

CONCLUSIONS

Glutathione has long been known to be a vital antioxidant, detoxifier, and an important component both in prokaryotic and eukaryotic cells. However, all its roles have yet to be completely understood. The fact that GSH is not present in many subgroups of eubacteria, and archaebacteria clearly highlights that bacteria can survive without this antioxidant. A blend of genetic and biochemical approaches to study the function of GSH in bacteria have established many of the roles of this simple tripeptide thiol. GSH plays a major role during taxing conditions that bacteria have to endure, such as osmotic and oxidative stresses, excessive production or exposure to toxins, inhibitory effects of chlorine compounds and acidity. Also GSH is part of the glutaredoxin pathway that supplies reducing equivalents to ribonucleotide reductase which is responsible for the reduction of ribonucleotides to deoxyribonucleotides. While glutathionylation appears to be a common mode of redox regulation in eukaryotes, in bacteria only a few proteins, like MetE, PAPS reductase, and possibly OxyR, have been found to undergo glutathionylation. Many critical questions such as why proteins undergo glutathionylation and how the functions of glutathionylated proteins vary from their normal counterparts have yet to be completely understood. The mechanisms that maintain the level of GSH in the cell, and the consequences of deregulating GSH synthesis with respect to aberrant glutathionylation or perturbation of the cellular redox balance are also not well understood. Studies in our laboratory involve the application of high throughput screening technologies such as FACS for detection of GSH in single cells thus enabling genetic analysis of bacterial populations based on intracellular GSH levels. We hope that this and other approaches not described in this review will help complete our understanding of the role of this fascinating molecule and also open the way for novel biotechnology applications.

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ABBREVIATIONS

DCM, dichloromethane; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GCS, γ-glutamylcysteine synthetase; GGT, γ-glutamyl transpeptidase; GS, glutathione synthetase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; HOCl, hypochlorous acid; HPLC, high performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; mBBr, monobromobimane; NH_2Cl, monochloroamine; PAPS, 3′-phosphoadenylsulfate; P_i, inorganic phosphate.

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