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Glutathione Reductase Is Not Required for Maintenance of Reduced Glutathione in *Escherichia coli* K-12

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Seven independently isolated glutathione reductase-deficient (*gor*) *Escherichia coli* mutants were found to have an in vivo glutathione redox state that did not significantly differ from that of the parental strain, 98 to 99% reduced. Strains containing both a *gor* mutation and either a *trxA* mutation (thioredoxin deficient) or a *trxB* mutation (thioredoxin reductase deficient) were able to maintain a 94 to 96% reduced glutathione pool, suggesting that glutathione can be reduced independently of glutathione reductase and thioredoxin reductase.

Glutathione reductase (NAD(P)H:oxidized-glutathione oxidoreductase; EC 1.6.4.2) is a nearly ubiquitous enzyme and has been studied in numerous organisms, including mammals (20) and yeasts (3) and other microorganisms (2, 7, 10). In *Escherichia coli*, glutathione reductase, glutathione and glutaredoxin have been shown to couple NADPH oxidation to reduction of ribonucleotides (8, 14) and sulfate (22) as well as methionine sulfoxide (9). This reducing system may serve as an alternative to thioredoxin and thioredoxin reductase, which can also be used in these reactions. Because mutants deficient in glutathione biosynthesis (1, 11) grow without supplemented glutathione, the latter does not appear to be essential for normal cell maintenance. In addition, mutants deficient in glutathione reductase activity have been isolated (2, 7, 10) and mapped (7). One such mutant, JF420 (*gor-1* [10]), was isolated in a selection for mutants that had increased sensitivity to the thiol oxidant, diamide (17). JF420 has less than 4% of parental glutathione reductase activity yet is able to maintain the pool of glutathione highly reduced. Another mutant, JM2267 (*gor*::Mu *cts* Ap [7]), was isolated without selection after random insertional mutagenesis with Mu *cts* Ap. Two strains that are deficient in glutathione reductase activity and were isolated in a selection for mutants with increased sensitivity to hydrogen peroxide relative to a catalase-deficient parental strain have also been reported (2).

Since JF420 is the only mutant characterized with respect to in vivo glutathione pool redox state and may contain a glutathione reductase that functions in vivo but not in vitro, five additional glutathione reductase mutant strains, independently isolated in the same selection as JF420, plus the *gor*::Mu *cts* Ap mutant strain (7), were characterized.

Table 1 shows the mutants used in this study. Glutathione reductase assays of three of these mutants (JF420, JF422, and JF424), as well as JM2267, had rates of NADPH oxidation that did not significantly differ from that observed when no substrate was added (Table 2). JF421, JF423, and JF425 have low but detectable glutathione reductase activities. The redox state of the glutathione pool in these strains was then measured. In all seven mutant strains, including JM2267 (the insertionally inactivated *gor* mutant), the glutathione pool remained highly reduced (Table 2). These results indicate that another thiol-reducing system may be reducing oxidized glutathione. The thioredoxin system would appear to be the logical candidate, since the thioredoxin and glutathione systems are alternate pathways used by several enzymes as sources of electrons (9, 14, 22). Strains deficient in both glutathione reductase activity (*gor-1*) and either thioredoxin activity (*trxA*) or thioredoxin reductase activity (*trxB*) were constructed. JF435 (*gor-1 trxA*) was constructed by transducing JF420 to kanamycin resistance with P1 grown on BH2012, a strain with Tn*5* integrated into the *ilvC* locus, which is closely linked to *trxA*. Kanamycin-resistant

TABLE 1. Bacterial strains[a]

Strain	Sex	Genotype	Source (reference)
AB1157	F⁻	*thr-1 leu-6 thi-1 lacY-1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 sup-37*	(16)
BH10	F⁻	*cysB metB rpsL thyA*(Ts) *trxB upp udk drm*	*cysB* derivative of KK1048 (8)
BH51	F′123	BH10 with F′123 transferred from BH1100	This laboratory
BH1100	F′123	*thi-1 pyrD34 his-68 trp-45 recA1 mtl-2 xyl-7 malA1 galK35 strA11B* λ^r, λ⁻	(18)
BH2012	F⁻	MC1061 *leu*⁺ *lac*⁺ *metE46 argH1 trxA7004 ilvC*::Tn*5*	This laboratory
JF420	F⁻	AB1157 *gor-1*[b]	(10)
JF421	F⁻	AB1157 *gor-2*	This paper
JF422	F⁻	AB1157 *gor-3*	This paper
JF423	F⁻	AB1157 *gor-4*	This paper
JF424	F⁻	AB1157 *gor-5*	This paper
JF425	F⁻	AB1157 *gor-6*	This paper
JF431	F⁻	AB1157 *trxA7004 ilvC*::Tn*5* Kn^r	This paper
JF432	F⁻	AB1157 *trxB gal*⁺	This paper
JF435	F⁻	JF420 *trxA7004 ilvC*::Tn*5* Kn^r	This paper
JF440	F⁻	JF420 *trxB gal*⁺	This paper
JM2267	F⁻	*lac ara* Δ(*his-gnd*) Str^r *gor*::Mu *cts* Ap	(7)
MC1061	F⁻	*araD139* Δ(*ara-leu*)*7697* Δ-*lacX74 galU galK hsr hsm*⁺ *rpsL*	(4)

[a] Cells were grown in minimal medium (6) plus required supplements, except for some enzyme assays and diamide experiments, in which cells were grown in Luria broth (19). In experiments involving ^{35}S labeling, the sulfate concentration was reduced to 0.1 mM.

[b] The approach used for isolation of glutathione reductase-deficient strains was that of Apontoweil and Berends (1).

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TABLE 2. Glutathione reductase activity and glutathione pool redox state in mutant and parent strains

Strain	Activity[a]	% GSH[b]
AB1157	35.0	99.8 ± 0.1
JF420	0.45	99.0 ± 0.4
JF421	3.1	99.4 ± 0.2
JF422	1.2	99.1 ± 0.1
JF423	1.9	99.1 ± 0.2
JF424	1.1	99.7 ± 0.1
JF425	7.0	99.4 ± 0.1
JM2267	1.0	99.6 ± 0.01

[a] Activity is given in nanomoles of NADPH oxidized per minute per milligram of protein. The rate of NADPH is the rate observed after oxidized glutathione addition minus the rate before addition of oxidized glutathione. The latter rate was approximately 2.0 nmol min^{-1} mg of $protein^{-1}$.

[b] Glutathione was labeled, extracted, and separated essentially as described by Hibbered et al. (13). Percent glutathione (GSH) was calculated as follows: % GSH = [(nmol of GSH) (nmol of GSH + nmol of GSSG)$^{-1}$] × 100, where GSSG is oxidized glutathione.

transductants were then screened for the inability to form T7 plaques upon infection (5). JF440 (*gor-1 trxB*) was constructed by conjugative transfer of the thioredoxin reductase mutant allele from BH51 into JF420 by transfer of the closely linked *gal*$^+$ allele (12). *gal*$^+$ derivatives of JF420 were then assayed for thioredoxin reductase activity (8). Retention of the mutant *gor-1* allele in these constructions were confirmed by glutathione reductase assay (11). Serving as controls, *trxA* and *trxB* derivatives of AB1157 were constructed in the same manner. The redox state of the glutathione pool of all these double mutants was highly reduced (Table 3, 0 min column). JF435 and JF440, however, appear to have a slightly increased level of oxidized glutathione (4 and 6%, respectively) compared with JF420 (2%), which could indicate that the thioredoxin system may be involved in reducing glutathione in JF420.

JF420 was found to be more sensitive to the thiol oxidant diamide than was its parent, AB1157, at all diamide concentrations tested, and eventual recovery of both parent and mutant from oxidative stress occurred only when the redox state of the glutathione pool returned to the untreated level (10). JM2267 (*gor*::Mu *cts* Ap) behaves in the same manner as JF420 under these conditions (data not shown). To determine whether the thioredoxin system plays a role in recovery from oxidative stress, we added 100 μM diamide to radiolabeled, exponentially growing cultures of the double mutants JF435 and JF440 as well as their respective parental strains. The redox state of the glutathione pool and the growth lag of these strains was determined (Table 3). After addition of diamide, the redox states of the *trxA* and *trxB* derivatives of AB1157 did not differ from that of AB1157 (99% glutathione). The *gor-1* mutant JF420, as well as the double mutants JF435 and JF440, exhibited a significantly decreased redox level in the glutathione pool 5 min after diamide addition. At 60 min after diamide addition, however, all three strains had a glutathione pool redox state of over 90% glutathione. Although both JF435 and JF440 resumed exponential growth later than did JF420 and their glutathione redox state remained lower than that of JF420 even after growth resumption, these results suggest that the thioredoxin system does not play a major role in the recovery of a *gor-1* mutant from oxidative stress.

Neither JF435 nor JF440 are able to grow at 42°C in either Luria broth or in minimal medium supplemented with either cysteine or glutathione. At 30°C, the addition of either cysteine or glutathione increases the growth rate of these double mutants on solid minimal medium (data not shown). These phenotypes have been observed for other mutants blocked in both glutathione biosynthesis and the thioredoxin pathway (10). Since growth of JF435 or JF440 stops immediately after a shift to 42°C, the redox state of the glutathione pool in the double mutants as well as the *trxA*, *trxB*, *gor-1*, and nonmutant parental strains was followed after a 42°C shift. The double mutants exhibited a slight shift in the redox state of the glutathione pool, which was then corrected to the original level (Table 4). Thus, growth inhibition at 42°C is apparently not due to a change in the redox state of the glutathione pool in these double mutants.

A previous report (10) that JF420 has no glutathione reductase activity but is able to maintain an intracellular reduced glutathione pool has now been extended to include six additional independently isolated mutant strains, including one that was derived by insertion of a transposon into the gene for glutathione reductase. The latter event would have a low probability of producing a mutant without measurable in vitro activity but with maintenance of in vivo activity. These results indicate that *E. coli* must have an active disulfide reductase system that can serve in place of glutathione reductase to keep low-molecular-weight disulfides reduced. One such system is the well-characterized thioredoxin and thioredoxin reductase system (21). Although thioredoxin prefers high-molecular-weight disulfides, it reduces low-molecular-weight disulfides as well (15). We found, however, that double mutants defective in both glutathione reductase and either thioredoxin or thioredoxin reductase were able to keep the in vivo glutathione pool in the reduced state, indicating that an additional powerful disulfide reducing system must be functioning in these strains. Holmgren has shown that lipoamide and lipoamide dehydrogenase can reduce thioredoxin in vitro (15). Thus, this system may serve as an alternate pathway for cellular disulfide reduction and could explain the viability of the *gor-1 trxB* double mutant, JF440. In JF435, the *gor-1 trxA* double mutant, lipoamide

TABLE 3. Glutathione pool redox state and growth lag of double mutants and parents after diamide addition[a]

Strain	% GSH[b] at following time (min) after diamide addition:			Growth lag (min)[c]
	0	5	60	
AB1157[d]	99.5 ± 0.4	98.8 ± 0.7	99.6 ± 0.1	<5
JF420	98.0 ± 0.1	33.9 ± 2.6	98.0 ± 0.7	12
JF435	96.0 ± 0.4	42.3 ± 4.3	91.0 ± 0.5	26
JF440	94.0 ± 0.9	60.0 ± 2.5	91.0 ± 4.3	45

[a] Diamide was added at a concentration of 100 μM.

[b] GSH, Glutathione. See Table 2, footnote *b*, for calculations.

[c] Lag was calculated by extrapolation of the slope of the renewed growth rate of the culture back to an optical density at 660 nm at the time of diamide addition.

[d] JF431 and JF432 values did not differ from those of AB1157.

TABLE 4. Glutathione pool redox state of double mutants and parents after temperature shift

Strain	% GSH[a] at following time (min) after shift to 42°C:			
	0	10	40	90
AB1157	99.5 ± 0.4	99.5 ± 0.1	99.3 ± 0.2	99.3 ± 0.2
JF420	98.0 ± 0.1	99.0 ± 0.1	99.0 ± 0.1	98.0 ± 0.1
JF435	96.0 ± 0.4	91.6 ± 0.4	94.0 ± 2.2	96.0 ± 1.3
JF440	94.0 ± 0.9	89.0 ± 0.8	97.0 ± 0.4	97.0 ± 0.2

[a] GSH, Glutathione. See Table 2, footnote *b*, for calculations.

and lipoamide dehydrogenase, would presumably reduce glutaredoxin in an analogous way. None of these double mutants, however, are able to grow at 42°C, for a reason we presently do not understand.

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