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Construction and characterization of glutaredoxin-negative mutants of *Escherichia coli*

(ribonucleotide reductase/gene replacement/thioredoxin/glutathione/DNA synthesis)

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ABSTRACT **Deoxyribonucleotides, the precursors of DNA, are formed *de novo* by ribonucleotide reductase, and *in vitro* thioredoxin or glutathione plus glutaredoxin have been isolated as hydrogen donors. The *in vivo* hydrogen donor for ribonucleotide reductase is not known. To study this, the *Escherichia coli* glutaredoxin gene (255 base pairs) was inactivated by inserting a 2-kilobase kanamycin-resistance fragment into the coding sequence of the cloned gene. The inactivated gene was inserted into the *E. coli* chromosome and mapped to about 18.5 min. A gene replacement technique was used to obtain a strain, A407, that lacked glutaredoxin by radioimmunoassay and by enzymatic assay with ribonucleotide reductase. Glutaredoxin was found not to be essential for viability of *E. coli*. Thioredoxin is also not essential for viability, as had been shown earlier, but a double mutant lacking glutaredoxin and thioredoxin could not be obtained by P1 transduction on a defined medium, indicating that either thioredoxin or glutaredoxin is essential. In rich medium, very slowly growing, unstable transductants were obtained that at high frequency gave rise to better growing cells. One such isolate, A410, was shown to still lack glutaredoxin and thioredoxin.**

Escherichia coli cells do not take up deoxyribonucleotides (1) nor can they phosphorylate deoxyribonucleosides other than thymidine (2); their only source of the three other deoxynucleotides required for DNA synthesis is the reduction of ribonucleoside diphosphates, by way of ribonucleoside diphosphate reductase. As these observations suggest and the isolation of conditionally lethal mutants confirms (3–5), ribonucleotide reductase is essential in *E. coli*. The hydrogen donor for ribonucleotide reductase was originally identified as thioredoxin, a small (12 kDa) dithiol protein (6, 7), which, in turn, is reduced by NADPH and thioredoxin reductase (8). Subsequently, a second small (10 kDa) dithiol protein termed glutaredoxin (9, 10) was discovered in a mutant *E. coli* that lacked thioredoxin (11) but had a fully active NADPH-dependent ribonucleotide reduction system (9). Glutaredoxin is reduced by glutathione, which, in turn, is reduced by NADPH and glutathione reductase (12). These relationships are diagramed in Fig. 1.

Thioredoxin is more abundant in *E. coli* than glutaredoxin, but the latter has a lower K_m for ribonucleotide reductase (10, 12). These two proteins play a role in sulfate (13) and disulfide (12) reduction, but except in the reduction of methionine sulfoxide, where glutaredoxin appears unable to substitute for thioredoxin (14), the relative contribution of thioredoxin and glutaredoxin for ribonucleotide reduction, or for these other reactions, is not yet known. The role of glutaredoxin in *E. coli* could be analyzed by using mutants in the glutaredoxin (*grx*) gene, but no mutants have yet been reported nor has the *grx* gene been mapped on the *E. coli* chromosome.

In previous work (15, 16), the *grx* gene was cloned and its nucleotide sequence was determined. Cells containing the 255-base-pair (bp) *grx* structural gene flanked by about 280 (5′) and 500 (3′) bp cloned in pBR322 overproduced glutaredoxin 10- to 20 fold, demonstrating that the cloned gene was functional (15).

Since no simple procedure exists for the isolation of *grx*-negative mutants, we have used a combination of molecular biologic and genetic techniques to map the gene and isolate a *grx* mutant. We have also combined the mutation in the *grx* gene with a thioredoxin (*trxA*) null mutant (14) with unexpected consequences.

MATERIALS AND METHODS

Bacterial Strains, Media, and Genetic Techniques. Bacterial strains used in this study are listed in Table 1. P1 transductions were done as described (19), except that phenotypic expression took place *in situ*; 0.3 ml of tetracycline (2.5 mg/ml) was inserted under the agar just after the cells had been spread. Cotransduction of linked markers was determined by replica plating unless otherwise indicated. Transformations were done according to the $CaCl_2$ procedure (20). Cultures were grown in defined medium containing 0.4% glucose and 5 μg of thiamine per ml, with or without 0.2% Casamino acids (19), or in fortified broth (FB medium) (21). Cells were plated on Ty plates (21).

Plasmid Constructions. The plasmid pBRgrx, previously called pBR322ECG (15), contains the entire *grx* gene on a 1.0-kilobase (kb) *Hae* III fragment. A plasmid, pPMR28, that specifies trimethoprim resistance and contains a functional *grx* gene was constructed by cloning the *grx*-containing *Eco*RI–*Bam*HI fragment from pBRgrx in place of an *Eco*RI–*Bgl* II fragment in the plasmid pCY2, a high-copy, *polA*-independent plasmid (22).

The plasmid pBRgrx::kan was constructed by cloning the 2-kb *Bam*HI fragment from pSK101, which contains the kanamycin-resistance gene (23) into *Bgl* II-digested pBRgrx. There are two *Bgl* II sites in pBRgrx, both within the *grx* coding region (15). In the isolate selected for further study, the *kan*^{r} fragment had replaced the 84-bp *Bgl* II fragment such that *grx* and *kan* were transcribed in the same direction.

DNA manipulations were done by standard methods, following the recommendations of the manufacturers. Restriction emzymes were from New England Biolabs or Bethesda Research Laboratories. Plasmid DNA was prepared as described (19).

Preparation of Extracts and *in Vitro* Assays. Cultures of A407, A408, and A410 cells were grown at 37°C with shaking in 400 ml of FB medium. The growth was monitored and

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Abbreviations: ^{r}, resistant; ^{s}, sensitive; Kan, kanamycin; Tet, tetracycline; Amp, ampicillin.

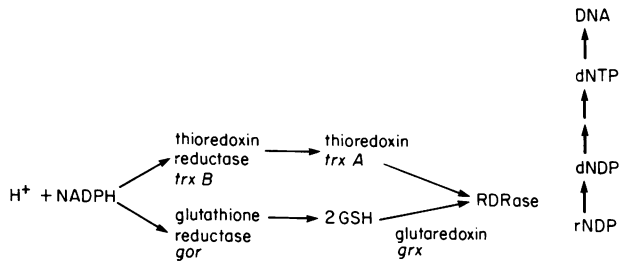

FIG. 1. Thioredoxin- and glutaredoxin-dependent system of ribonucleotide reduction. RDRase is ribonucleoside diphosphate reductase; GSH is reduced glutathione. The names of the genes encoding the relevant enzymes are given in italics.

cells were harvested by centrifugation at an A_{650} of about 0.7. The cells were sonicated with five times their weight of 50 mM Tris·HCl/1 mM EDTA, pH 7.5, and crude cell-free extracts were prepared by centrifugation at 10,000 × *g* (Sorvall SS-34) for 30 min. The extracts, containing 16 mg of protein per ml, were used immediately for assays.

A radioimmunoassay (11) using a rabbit anti-glutaredoxin antibody (16) was used to measure glutaredoxin in crude extracts. Each tube in the assay contained 4 ng of ^{125}I-labeled glutaredoxin in 30 μl, 100 μl of a 1:2000 dilution of anti-glutaredoxin (10 nmol/ml), and either 100 μl of crude extract diluted with 1 mg of bovine serum albumin per ml/0.15 M NaCl/0.01 M potassium phosphate, pH 7.0 (PBS/bovine serum albumin), or 100 μl of homogenous glutaredoxin (10) in PBS/bovine serum albumin. After incubation for 4 hr at 37°C, 100 μl of a 1:5 dilution of sheep anti-rabbit gamma globulin was added and incubation was continued for 16 hr at 4°C. After centrifugation at 5000 × *g* for 20 min, radioactivity in the immune precipitates was determined with a γ-counter.

Crude extracts from A407, A408, and A410 cells were heated to 85°C for 5 min, and precipitated protein was removed by centrifugation. The content of glutaredoxin or thioredoxin in the heated extracts, which contained 3 mg of protein per ml, was determined by assay with excess purified *E. coli* ribonucleotide reductase (9), as described (12). With 4 mM glutathione, 1 mM NADPH, and 2 μg of glutathione reductase per ml, only glutaredoxin activity (12) is measured. Supplementation with thioredoxin reductase was used to determine the sum of the activities of glutaredoxin and thioredoxin as hydrogen donors for ribonucleotide reductase.

RESULTS

Despite intensive efforts using conventional techniques, no glutaredoxin mutants had previously been isolated (A.H., unpublished results), so the gene was mapped by the method of Greener and Hill (18), which derives from the inability of colE1-derived plasmids such as pBR322 to replicate in *polA* mutant cells. They found that *polA* strains could be transformed by such plasmids only if the plasmid contained *E. coli* DNA and that transformants contained plasmid integrated into the chromosome at the position of the homology; they used the antibiotic-resistance marker encoded by the plasmid to map its chromosomal location in matings between *polA* Hfr and *polA* F$^-$ strains.

These observations were later extended to develop procedures for gene replacement (24, 25). The plasmid integrants have two copies of the DNA originally cloned, separated by the vector portion of the plasmid as diagramed in Fig. 2*A*; a mutant copy of the plasmid gene can be constructed (frequently by cloning an antibiotic-resistance gene—*kan*^{r} in Fig. 2—within the donor gene). After the plasmid has been integrated into the chromosome, segregants that have lost the plasmid marker (i.e., *amp*^{r}) along with the wild-type copy of the gene, leaving the mutant allele (i.e., *kan*^{r}) in the chromosome (Fig. 2*B*, *c*), are sought.

Mapping the *grx* Gene. The *grx* gene cloned in pBR322 contains two unique *Bgl* II sites within the coding region (15). A complete kanamycin-resistance gene was cloned in place of the small *Bgl* II fragment, creating pBRgrx::kan. This plasmid was introduced into an HfrC *polA1* mutant strain, creating polA1::pBRgrx::kan, where :: indicates that the plasmid has inserted into the bacterial chromosome and that the *kan* gene has been inserted into the *grx* gene. Then the *kan*^{r} marker was mapped in matings with an F$^-$ *polA1* recipient. The *kan*^{r} gene was located between 15 and 28 min on the *E. coli* chromosome (data not shown).

A number of strains have been isolated that contain Tn*10* (*tet*^{r}) transposons at various positions between 15 and 28 min, and these were used to further localize the *kan*^{r} (and *grx*) gene. Initially, a Tn*10* located at 19–20 min (*zbj*-

Table 1. Bacterial strains

Strain	Genotype	Source or ref.
K38	HfrC (λ)	S26 RIE sup$^+$
K937	*serC13 (serS14 serS16)*	KL282 (K. Low)
K946	*zbj-1230*::Tn*10*	CGSC no. 6392
K1105	*zbi*::Tn*10*	17
A307	K38 Δ*trxA*	14
A401	HfrC *polA1*	C. Hill
A402	HfrC polA1::pBRgrx::kan	This study
A403	A402 *zbj-1230*::Tn*10*	This study
A405	F$^-$ *trpA lysA argH polA1 sm*^{r}	18
A406	A402 *zbi*::Tn*10*	This study
A407	K38 *zbi*::Tn*10 grx*::*kan*	This study
A408	K38 *zbi*::Tn*10*	This study
A409	K38 *zbi*::Tn*10* grx::kan-pPMR28	Amp^s, Kan^r
A410	K38 Δ*trxA zbi*::Tn*10 grx*::*kan* X	This study

Amp, ampicillin; Kan, kanamycin; ^{s}, sensitive; ^{r}, resistant.

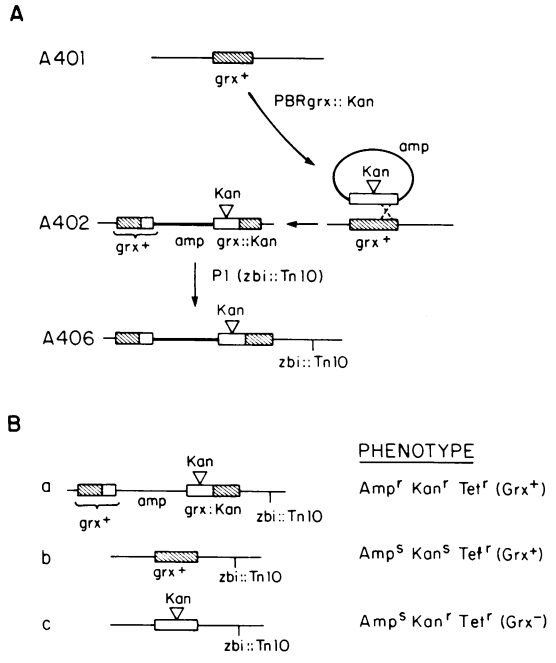

FIG. 2. Outline of gene mapping and gene replacement. (*A*) Integration of pBRgrx::kan into the chromosome by way of a single cross-over event and introduction of the closely linked marker *zbi*::Tn*10*. (*B*) Possible configurations of *polA*$^+$ strain transduced to tetracycline resistance (Tet^r), using A406 as donor: *a* is at least partially lethal, *b* arises if the cross-over that removes the plasmid occurs in the same interval as the event by which it had integrated, and *c*, the desired product, arises if the second cross-over occurs on the other side of the *kan* gene as the first.

1230::Tn*10*) was introduced into the polA1::pBRgrx::kan strain by P1 transduction. The *tet* and *kan* markers cotransduced, indicating that the integrated pBRgrx::kan and the transposon were linked. These markers were also mapped with respect to a conventional marker, serC, located at 20 min; *kan*^{r} and serC$^+$ cotransduced (8%), indicating that the pBRgrx::kan plasmid had integrated into the chromosome within about 1 min of serC. A Tn*10* insertion, *zbi*::Tn*10*, located at 18.5 min (17) was introduced into the polA1::pBRgrx::kan strain by transduction, and, as before, the linkage between the *tet*^{r} and *kan*^{r} markers was determined; 95% of the Tet^r transductants were Kan^r, indicating that the integrated pBRgrx::kan plasmid, and thus the *grx* gene itself, is located at about 18.5 min. It is unlinked to the thioredoxin gene located at 84 min (26).

Gene Replacement of *grx*$^+$ by *grx*::*kan*. A diagram outlining the scheme for gene replacement is shown in Fig. 2. Fig. 2*A* shows the construction of the donor strain, A406, and Fig. 2*B* indicates the expected products from transduction into a *polA*$^+$ recipient strain. Note that the presence of the integrated plasmid can be detected by determining whether the transductants are Amp^r. A406 was used as donor to transduce K38-pPMR28 to Tet^r, and the transductants were scored for cotransduction of Kan^r and for loss of the integrated plasmid. Of six Tet^r, Kan^r transductants of K38-pPMR28 tested, one was *amp*^{s}; it was saved as A409, a presumptive *grx*::*kan* gene replacement. The recipient strain also contained pPMR28, which provides glutaredoxin, so that the *grx*::*kan* gene replacement could be isolated even if glutaredoxin proved to be essential for viability of *E. coli*.

Is Glutaredoxin Essential to Viability? A409 was used as donor to transduce K38 or K38-pPMR28 to Tet^r, and Kan^r was scored. Similar numbers of Tet^r transductants were obtained in the two recipient strains, and cotransduction frequencies (Tet^r, Kan^r) were also similar (Table 2). Thus, it is not necessary to provide glutaredoxin in trans. One Tet^r Kan^r transductant of K38 (*zbi*::Tn*10* *grx*::*kan*) was saved as A407. As documented below, A407 lacks glutaredoxin; hence, the gene replacement was successful, the *kan*^{r} marker can legitimately be referred to as *grx*::*kan*, and glutaredoxin is nonessential for viability in *E. coli*.

Viability of a *trxA-grx* Double Mutant. Thioredoxin is an abundant (*ca.* 10,000 molecules per cell) protein (7) originally identified as a cofactor required for ribonucleotide reductase activity *in vitro* (6). The discovery that it was nonessential in *E. coli* (11, 25) was surprising until the existence of glutaredoxin (*ca.* 100–1000 molecules per cell, and variable) (27) was demonstrated by isolating this second cofactor from cells that lacked thioredoxin (9, 10), and the concept of a "back-up" system for protein disulfide reduction was developed. It seemed likely that though glutaredoxin and thioredoxin might be able to substitute for one another in reducing the essential enzyme ribonucleotide reductase, the absence of both proteins would be lethal.

To test this, the isogenic strains K38 (*trxA*$^+$) and A307 (Δ*trxA*) (14) were transduced to Tet^r and scored for *grx*::*kan* (Kan^r) by using A407 (*zbi*::Tn*10* *grx*::*kan*) as donor. Table 3 shows that no Δ*trxA-grx*::*kan* transductants were obtained. K38 and A307 could be transduced to Tet^r at equal frequency when a P1(*zbi*::Tn*10* *grx*$^+$) donor was used; however, with the P1(*zbi*::Tn*10* *grx*::*kan*) donor, the Tet^r frequency in A307 was reduced to 6% of the *trxA*$^+$ control. None of the Tet^r transductants of A307 were Kan^r, whereas 94% of the K38 Tet^r were. Thus, only those Δ*trxA* *zbi*::Tn*10* cells in which the *grx*::*kan* allele had not been incorporated survived, and the Δ*trxA* *grx*::*kan* double mutant must be nonviable.

Table 2. *grx* is not an essential gene

P1 donor marker	Recipient	No. of Tet^r	No. of Tet^r, Kan^r (%)*
zbi::Tn*10*	K38	14	—
zbi::Tn*10* *grx*::*kan*	K38	182	73† (40)
zbi::Tn*10*	K38 + pPMR28	14	—
zbi::Tn*10* *grx*::*kan*	K38 + pPMR28	115	29 (25)

*Tet^r, Kan^r transductants were selected simultaneously by underlayering with Tet and Kan, which underestimated the cotransduction frequency compared to scoring by replica plating.
†A single transductant was saved as A407.

These P1 transductions were routinely done by selecting on a defined medium that contained glucose and Casamino acids (19). It seemed possible that this medium might lack some factor essential for survival of a *trxA-grx* double mutant. Consequently, the transduction was repeated, selecting Tet^r transductants on rich (Ty) plates. Under these conditions, using A407 as donor, the transduction frequency of A307 to Tet^r was higher (62% the K38 level) and some Kan^r cotransductants were detected. These Tet^r Kan^r colonies grew very slowly, requiring about 2 days of growth at 37°C to make extremely small colonies, much smaller than the Δ*trxA* *grx*$^+$ control. The small Δ*trxA* *grx*::*kan* colonies spontaneously (and at high frequency) gave rise to cells that formed larger colonies. These larger colony-formers seemed stable, and one was saved as A410. A410 lacks thioredoxin and glutaredoxin by genetic criteria. It fails to support growth of filamentous phage that require thioredoxin for assembly (28, 29), indicating that it lacks thioredoxin. It can be used as donor to transduce a *trxA*$^+$ strain or a Δ*trxA* strain containing a *grx*$^+$ plasmid (pPMR28) to Tet^r Kan^r, but it cannot transduce a Δ*trxA* strain to *grx*::*kan*, selecting on defined medium. Furthermore, as confirmed below, A410 lacks the thioredoxin and glutaredoxin proteins. Thus, an initial Δ*trxA* *grx*::*kan* transductant is barely viable on complex medium, but mutants that grow better are rapidly selected. The nature of this mutation, herein called X, has not been determined. The failure of the Δ*trxA* *grx*::*kan* double mutant to survive when A410 is used as the donor indicates that the X mutation is not closely linked to the *grx* locus.

Confirmation of Gene Replacement. To confirm that the gene replacement had been successful and that strains A407 and A410 lacked glutaredoxin, a radioimmunoassay of crude extracts from cells was performed. Fig. 3 shows that extracts prepared from these strains do not contain material that competes with ^{125}I-labeled glutaredoxin for glutaredoxin-specific antibodies. By contrast, an extract prepared from A408 (*grx*$^+$ *zbi*::Tn*10*), an isogenic control strain, contained glutaredoxin. Comparison with a standard curve obtained by using known amounts of purified glutaredoxin indicated that the control extract contained about 1.2 μg of glutaredoxin per ml, in good agreement with previous estimates for other wild-type strains of *E. coli* (15, 27).

The glutaredoxin and thioredoxin contents of the extracts were also determined enzymatically. Thioredoxin was assayed by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) method (11); in the presence of added NADPH and thioredoxin reductase, the A407 and A408 extracts reduced DTNB, whereas the A410 extract did not (data not shown). Glutaredoxin activity was measured in an assay that coupled it to the reduction of [^{3}H]CDP to dCDP by ribonucleotide reductase. Table 4 shows that in the absence of extract, little

Table 3. Viability of Δ*trxA* *grx*::*kan* double mutant

P1 donor marker	Recipient	No. of Tet^r	No. of Tet^r, Kan^r (%)
zbi::Tn*10*	K38 (*trxA*$^+$)	30	—
zbi::Tn*10* *grx*::*kan*	K38	157	148 (94)
zbi::Tn*10*	A307 (Δ*trxA*)	27	—
zbi::Tn*10* *grx*::*kan*	A307	10	0

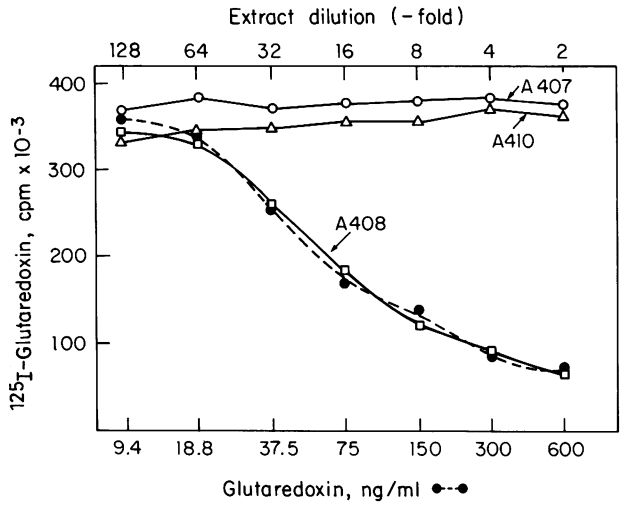

FIG. 3. Determination of glutaredoxin by radioimmunoassay in crude extracts from A407 (○), A408 (□), or A410 (△). A standard of glutaredoxin (●) is shown. All incubations contained 100 μl of either standard glutaredoxin or dilutions of crude extracts from cells. The ^{125}I-labeled glutaredoxin in the precipitate after additional excess sheep anti-rabbit gamma globulin is shown.

dCDP was generated by glutathione and ribonucleotide reductase alone, whereas addition of 5 pmol of purified glutaredoxin gave good activity. The A408 extract was active in this assay, whereas there was essentially no stimulation by the A407 or A410 extracts, confirming the absence of glutaredoxin. Addition of thioredoxin reductase in this assay will measure the sum of glutaredoxin and thioredoxin. As seen from Table 4, the A407 and A408 extracts contained thioredoxin, but the A410 did not, confirming the absence of both glutaredoxin and thioredoxin.

Properties of *grx* Mutants. Strain A407 (*grx*::*kan*) has no obvious phenotype; it grew at the same rate as its wild-type parent in FB medium, with a doubling time of 24 min at 37°C, in minimal medium supplemented with Casamino acids (45 min), and in minimal medium lacking amino acids (60 min). The growth defect of strains that lack thioredoxin has been noted before (11, 25); A307 (Δ*trxA*) grew less well under each condition, with doubling times of 34, 58, and 90 min, respectively. A410 (Δ*trxA grx*::*kan* X, where X is the uncharacterized "suppressor" mutation) grew as well as A307 in FB medium (35 min), but in minimal medium supplemented with Casamino acids it had a doubling time of 113 min, and no detectable growth occurred in minimal medium that lacked amino acids. Glutathione, methionine, or cysteine effectively substituted for Casamino acids in supporting A410 colony formation on plates containing salts, glucose, and thiamine. Anaerobic growth on rich plates did not have a marked effect on the growth of any of these strains.

Table 4. Assay of glutaredoxin and thioredoxin in heated extracts with ribonucleotide reductase

Addition	Activity, nmol of dCDP per 20 min
None	0.98
A407 E1 H (20 μl)	1.95
A408 E1 H (20 μl)	7.93
A410 E1 H (20 μl)	1.47
A407 E1 H (20 μl) + TR (20 pmol)	9.96
A408 E1 H (20 μl) + TR (20 pmol)	11.21
A410 E1 H (20 μl) + TR (20 pmol)	1.15
Glutaredoxin (5 pmol)	20.19

Each tube contained (in a final volume of 120 μl) 33 mM Hepes buffer (pH 7.6), 10 mM $MgCl_2$, 1.3 mM ATP, 0.5 mg of bovine serum albumin per ml, 4 mM glutathione, 2 μg of yeast glutathione reductase per ml, 1.6 mM NADPH, 0.5 mM [^{3}H]CDP (57,000 cpm/nmol), and 15 μg of *E. coli* ribonucleotide reductase. Extract (E1 H), *E. coli* thioredoxin reductase (TR), or glutaredoxin was added as indicated. After incubation for 20 min at 37°C, reactions were terminated with 1 ml perchloric acid, and the amount of [^{3}H]dCDP was determined after acid hydrolysis and separation on columns of Dowex 50 (12).

DISCUSSION

A strain containing a null mutation of *grx*, the gene encoding glutaredoxin, was isolated by a gene replacement technique. This procedure, which requires that the gene be cloned on a plasmid, is especially useful in cases where no clear mutant phenotype can be predicted, as was the case for *grx*. The technique relies on a Campbell-type recombination event between the inactivated, plasmid-borne gene and its chromosomal homologue, followed by a second recombination event to remove the plasmid and wild-type gene copy. Cells that have undergone the first recombination event, thereby integrating the plasmid, are easy to isolate by selecting antibiotic resistance. Identification of cells that have undergone the second recombination event is more difficult. Segregants have been found by screening for the spontaneous loss of the vector-encoded antibiotic-resistance gene (30), by selecting against the vector-resistance gene (24), which is possible for Tet (31), or by transducing into a *polA*$^+$ strain (25). The latter method relies on the deleterious effects of an active high-copy number replicon in the chromosome (32).

The segment of bacterial DNA contained in pBRgrx::kan was smaller than those previously used for gene replacement, containing 349- and 618-bp segments flanking the *kan*^{r} gene. Although spontaneous segregation has been reported at frequencies between 0.1% and 1.0% (30), no spontaneous *grx*::*kan* (*amp*^{s}) segregants were found among >9000 colonies screened; this failure is probably due to the small size of the regions available for homologous recombination. A recombinant that had lost the wild-type *grx* gene copy and the plasmid was, however, easily identified among the products of transduction into *polA*$^+$ cells. This suggests that recipient cells were effectively selected against due to hyperreplication of the integrated plasmid (32).

A closely linked chromosomal marker (*zbi*::Tn*10* in this case) was essential to the *grx*::*kan* gene replacement. Selecting for Kan resistance alone produced *ca.* 100 times as many transductants as when the Tn*10* marker (Tet^r) was selected, but these were apparently due to recircularization of the pBRgrx::kan plasmid, which, when not integrated into the chromosome, replicates normally in the *polA*$^+$ strain. Thus, by first selecting for transduction of the closely linked marker, only events that reflected chromosomal integration were detected.

By using this approach, we constructed an *E. coli* strain, A407, whose glutaredoxin gene has a deletion and an insertion. As expected, A407 cells do not contain glutaredoxin. This was shown by radioimmunoassay and by assays with ribonucleotide reductase. The A407 strain shows no phenotype, and consequently glutaredoxin does not play any irreplaceable role in ribonucleotide reduction or other processes required for viability of *E. coli* under laboratory conditions. Attempts to obtain a glutaredoxin-thioredoxin double mutant by transduction initially failed. In light of the viability of each single mutant, the inviability of the double mutant indicates that thioredoxin and glutaredoxin can substitute for one another in all essential reactions.

An unexpected, and so far poorly understood, result came from P1 transductions in which the transductants were plated on rich medium. It is not clear what component of the rich medium enabled the double mutant (*grx-trxA*) to survive

before it acquired the compensating mutation, X, that allowed it to grow with neither thioredoxin nor glutaredoxin. Thioredoxin and glutaredoxin are involved in sulfate reduction as well as in ribonucleotide reduction (12, 13); thus the compounds responsible may have been glutathione, cysteine, or methionine. It is also possible that there is some low-level utilization of exogenous deoxyribonucleosides or deoxyribonucleotides in these cells, just enough to let them grow very slowly.

Thioredoxin and glutaredoxin are the known hydrogen donors for ribonucleotide reductase. Since *E. coli* cannot use exogenous deoxyribonucleosides or deoxyribonucleotides other than thymidine (1, 2), it is not clear how A410 cells, which lack both donors and have acquired the X mutation, get deoxyribonucleotides with which to replicate their DNA. It is significant that they *can* grow on glucose minimal medium to which only glutathione, cysteine, or methionine has been added; therefore they must be dependent on endogenous nucleotides, and thus on ribonucleotide reductase as well. Several speculative possibilities exist regarding mutation X. Perhaps a cryptic ribonucleotide reductase system is activated that depends on neither thioredoxin nor glutaredoxin. An alternative gene that specifies a thioredoxin-like protein, as found in other species (7), could exist, and the X mutation might affect its expression; the inactivity of A410 extracts in the ribonucleotide reductase assay would then indicate either its heat instability or its inability to be reduced by glutathione. *E. coli* cells have several varieties of glutathione disulfide transhydrogenase activity (10) other than glutaredoxin, which might replace thioredoxin and glutaredoxin in the ribonucleotide reduction reaction. For example, reducing power could come from the dihydrolipoic acid residues of the pyruvate dehydrogenase complex, since lipoic acid can act as a disulfide reductant (33). Future experiments should, hopefully, clarify these points.

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