Di-iron YtfE Protein Is Necessary for the Repair of Stress-damaged Iron-Sulfur Clusters*

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DNA microarray experiments showed that the expression of the Escherichia coli ytfE gene is highly increased upon exposure to nitric oxide. We also reported that deletion of ytfE significantly alters the phenotype of E. coli, generating a strain with enhanced susceptibility to nitrosative stress and defective in the activity of several iron-sulfur-containing proteins. In this work, it is shown that the E. coli ytfE confers protection against oxidative stress. Furthermore, we found that the damage of the [4Fe-4S]2+ clusters of aconitase B and fumarase A caused by exposure to hydrogen peroxide and nitric oxide stress occurs at higher rates in the absence of ytfE. The ytfE null mutation also abolished the recovery of aconitase and fumarase activities, which is observed in wild type E. coli once the stress is scavenged. Notably, upon the addition of purified holo-YtfE protein to the mutant cell extracts, the enzymatic activities of fumarase and aconitase are fully recovered and at rates similar to the wild type strain. We concluded that YtfE is critical for the repair of iron-sulfur clusters damaged by oxidative and nitrosative stress conditions.

Iron-sulfur ([Fe-S]) clusters are very simple, almost ubiquitous, and evolutionary ancient prosthetic groups that are essential for the function of proteins involved in a wide range of biological processes, including electron transfer chains, redox and nonredox catalysis of reactions that underpin metabolic biological processes, including electron transfer chains, redox and nonredox catalysis of reactions that underpin metabolic pathways, and gene regulation, and as environmental sensors (1–3). Although in many cases in vitro assembly of [Fe-S] clusters and incorporation into apoproteins can occur spontaneously under reducing conditions and in the presence of ferrous iron and sulfide salts, in other cases [Fe-S] cluster biogenesis requires specific accessory proteins (2, 4–6). Furthermore, proteins that contain iron-sulfur clusters are one of the major targets of nitrosative and oxidative compounds that cause displacement of the iron atoms of the cluster and consequent malfunction of the protein/enzyme (7–10). In addition, the release of iron may further potentiate the effects of oxidative stress due to formation of the hydroxyl radical by the Fenton reaction (11). Under these conditions, enzymes of the dehydratase family that contain [4Fe-4S]2+ catalytic centers (e.g. aconitase and fumarase) are rapidly inactivated (12).

In Escherichia coli, three different machineries for iron-sulfur cluster biosynthesis were identified: the ISC (iron sulfur cluster) operon, the SUF (sulfur assimilation) operon, and the recently discovered CSD operon (reviewed in Refs. 2, 5, and 6). These systems are widely spread in nature, with the ISC system and their homologs present in bacteria and most eukaryotes and the SUF system present in bacteria, archaea, plants, and parasites. Despite the similarity of the biochemical activity exhibited by some of the proteins encoded by the isc and suf operons, which might suggest overlapping functions, the two systems seem to be adapted for different purposes. Regulation and phenotypic studies performed in E. coli led to the proposal that the ISC system acts as the housekeeping [Fe-S] cluster assembly system, whereas the SUF system constitutes a specific system for cluster assembly under oxidative stress and iron depletion conditions (5, 13). Nevertheless, in E. coli, both sufABCDSE and iscrSU LA operons are induced under oxidative and nitrosative stress conditions (14–17), and both Isc5 from E. coli and IscA from Azotobacter vinelandii seem to be also necessary for the repair of oxidatively damaged iron-sulfur clusters (13, 18).

In a previous study, we proposed that the di-iron protein E. coli YtfE could represent a novel system involved in the biosynthesis of iron-sulfur clusters, which is particularly important in cells subjected to stress conditions. In fact, expression of E. coli ytfE was found to be highly stimulated by nitrosative stress and iron starvation, and all examined iron-sulfur proteins had decreased activity levels in the strain lacking ytfE (16, 19). The goal of the present work is to evaluate the role of ytfE in oxidative stress and to determine whether YtfE is involved in the repair of degraded iron-sulfur clusters.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The strains used in this work were the wild type E. coli K-12 (ATCC 23716) and LMS4209 (K-12 ΔytfE) (16), and for plasmid manipulations, the E. coli XL2-Blue strain was used. The fumarase A-expressing vector (pGS57) (20) and aconitase B-expressing vector (pGS783) (21) were generous gifts of Jeffrey Green (University of Sheffield). For the complementation of the ytfE mutant strain, a fragment of 1.1 kb comprising the ytfE promoter and coding regions was amplified by PCR from E. coli K-12 genomic DNA, using the primers 5'-ATTAGTCGTGATTAGGATCCGATAGCCGT-3' and 5'-GGCTGTTTATTGGTAGAGAATTCGGCTGCTG-3' and was cloned BamHI/EcoRI into pUC18. The generated plasmid (ptyfE) was then used to transform the ΔytfE strain.
All cells were grown in Luria-Bertani (LB) medium, pH 7.0, at 37 °C and 150 rpm. Ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), and kanamycin (30 µg/ml) (all from Sigma) were used when necessary. For oxidative stress sensitivity assays, cultures grown under microaerophilic conditions (i.e., grown in closed flasks completely filled with medium) that reached an A₆₀₀ of 0.3 were treated with 2 mM hydrogen peroxide (Sigma) or left untreated, and the growth was followed for ~2 h. For fumarase expression, wild type and ytfE mutant cells were transformed with pGS57 and grown aerobically with 1 mM isofumarase expression, wild type and ytfE mutant strain expressing fumarase A or aconitase B were normalized for protein concentration and lysed by four freeze-thaw cycles, and aliquots of 300 µl were transferred to EPR tubes and frozen in liquid nitrogen as control samples. The cell lysates of the wild type and ytfE strains were treated with either 4 mM H₂O₂ or 150 µM nitric oxide (NO) and incubated at room temperature. At fixed time points, 300-µl aliquots were removed and frozen. NO treatment was done by the addition of a pure NO-saturated anaerobic water solution (~2 mM) prepared as described in Ref. 16. The EPR spectra were obtained as described above and at a temperature of 8–8.5 K.

Preparation of Holo- and Apo-YtfE—Recombinant E. coli YtfE protein was purified essentially as described in Ref. 19, as a mixture of monomer and dimeric forms, with 2 iron/monomer. To prepare the iron-depleted YtfE (apo-YtfE), 200 µM holo-YtfE was incubated with 10 mM EDTA and 2 mM dithiothreitol at 37 °C for 1 h in 20 mM Tris-HCl buffer, pH 7.5. The solution was then passed through a PD-10 desalting column (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer, pH 7.5.

Enzymatic Activity Assays—Enzymatic activities were determined in cell lysates prepared and treated in the same manner as the EPR samples. For the [Fe-S] cluster repair assays, 100 µg/ml tetracycline was added to the cell extracts 2 min before imposing the stress to inhibit de novo protein synthesis. H₂O₂ or NO stresses were stopped by the addition of 400 units of catalase or 40 µM hemoglobin (both from Sigma), respectively, and the enzyme activities were determined at fixed time points. Purified holo- or apo-YtfE proteins were added at 20 µM final concentration to the ytfE mutant cell extracts immediately after stopping the stress, and when appropriate, 1 mM 2,2′-dipyridyl was added before the protein. For the anaerobic incubation with ferrous iron, after the addition of catalase to the ytfE mutant cell extracts, the tubes were closed with Suba-seals, and the samples were deaerated under a flux of N₂ and thus maintained over the duration of the incubation. After 1–2 min, 5 mM dithiothreitol and 80 µM Fe(NH₄)₂(SO₄)₂ were added, and the time was set t = 0. Aconitase activity was determined by following the formation of NADPH through the indirect method described in Ref. 25. The samples were quickly thawed at room temperature and cleared by the addition of 0.5% (w/v) of sodium deoxycholate and a 1-min centrifugation at 12,000 × g. The samples were immediately inserted into Suba-sealed cuvettes, with deaerated 50 mM Tris-HCl, pH 7.7, 0.6 mM MnCl₂, 0.2 mM NADP⁺, and 1 unit of isocitrate dehydrogenase (Sigma), and the reactions were started by the addition of 30 mM sodium citrate. Fumarase activity was determined following the disappearance of fumarate as described in Ref. 26. The cell samples were quickly thawed at room temperature, cleared by the addition of 0.5% (w/v) sodium deoxycholate, and then digested in 50 mM sodium phosphate buffer, pH 7.3. The reactions were started by the addition of 10 mM fumarate and followed at 295 nm (εfumarate = 0.07 mM⁻¹ cm⁻¹) (26). Enzymatic activities were determined at 25 °C and are defined as units.

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The abbreviations used are: LB, Luria-Bertani; EPR, electron paramagnetic resonance; NO, nitric oxide.
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(μmol of product formed or consumed/min) per mg of total protein. The enzymatic activities were determined in duplicate from two independent cultures and are presented as averaged values with error bars representing one S.D.

RESULTS

YtfE Is Involved in the Response to Oxidative Stress Caused by Hydrogen Peroxide—To determine whether the ytfE gene could be involved in the E. coli response to oxidative stress, the growth of E. coli wild type and the ytfE mutant strains were compared upon treatment with H2O2. As shown in Fig. 1, the addition of 2 mM H2O2 caused a very small growth inhibition in the wild type strain, whereas the ΔytfE cells suffered growth impairment, clearly showing that the lack of ytfE results in a strain with higher sensitivity to hydrogen peroxide stress. The ytfE disruption mutant could be complemented by a clone containing the ytfE gene, since the complemented strain grew to final culture densities similar to that of the parent strain following treatment with hydrogen peroxide (Fig. 1).

The ytfE Mutant Has Higher Intracellular Iron Levels—We previously reported that the ytfE mutant strain is sensitive to iron limitation, that the ytfE gene is induced by iron starvation, and that Fur acts as a repressor in the transcription of ytfE (19). In addition, it was proposed that the repression of ytfE by YjeB may be responsible for the iron-dependent regulation of ytfE (27). In this work, the effect of streptonigrin, an iron-activated antibiotic whose toxicity increases with free iron, was evaluated on wild type and mutant strains in the absence and presence of the iron chelator 2,2′-dipyridyl (Table 1). The results clearly showed the higher sensitivity of the mutant strain to streptonigrin, even at a low antibiotic concentration (1 μg/ml), reflecting a higher amount of free iron in this strain. Whole cell EPR also demonstrated that the disruption of the ytfE gene led to a 2-fold increase in the intracellular levels of the free iron pool (data not shown).

Damage of [Fe-S] Clusters by Oxidative and Nitrosative Stress Is Accelerated in the Absence of ytfE—The E. coli strain disrupted in the ytfE gene displays lower resistance to stress conditions and diminished activity of iron-sulfur-containing enzymes (16, 19). Since [Fe-S] clusters are sensitive targets of both oxygen- and nitrogen-reactive species, we have evaluated the role of YtfE during damage caused by NO and H2O2 on two iron-sulfur proteins. To this end, E. coli wild type and ytfE mutant cells were transformed with plasmids pGS57 or pGS783 that contain the fumA and acnB genes, encoding for the [4Fe-4S]2+ cluster containing dehydratases fumarase A and aconitase B, respectively. The active [4Fe-4S]2+ forms of aconitase B and fumarase A are EPR-silent. Under exposure to oxidative stress conditions, the [4Fe-4S]2+ cluster converts to the enzymatically inactive [3Fe-4S]1+ form, which is EPR-detectable with a signal at g ~ 2.02 (12, 28). Prolonged exposure to stress conditions causes further degradation of the [3Fe-4S]1+ cluster to states that are also EPR-silent (13, 29). NO reacts with the iron atoms of the [Fe-S] clusters, forming iron-dinitrosyl complexes that are also EPR-detectable with a signal at g ~ 2.04 and are enzymatically inactive (7–9). Therefore, E. coli cells were grown under conditions that ensure the expression of the two enzymes, and the EPR signals of the [Fe-S] clusters and enzymatic activities of fumarase and aconitase were monitored under oxidative and nitrosative stress conditions (see “Experimental Procedures”).

Oxidative Stress—E. coli wild type and ytfE mutant cells expressing fumarase or aconitase were exposed to 4 mM H2O2, and EPR spectra were recorded at different times after the addition of the chemical. Figs. 2 and 3 show that in both strains, the addition of hydrogen peroxide induced the development of an EPR signal with g value of ~2.02, which corresponds to the degradation of the [4Fe-4S]2+ center to the enzymatically inactive intermediate [3Fe-4S]1+ form. However, the intensity and evolution of this signal and the time course of the activities of the two enzymes were different between the wild type and the mutant strain (Figs. 2 and 3).

In E. coli wild type cells overexpressing fumarase A, a signal at g ~ 2.02 was observed immediately after H2O2 exposure. Five minutes after the stress imposition, the signal exhibited a small increase, keeping the same intensity after 30 min (Fig. 2, A and C). Overexpression of fumarase A in ytfE-depleted cells gave rise to a more intense g ~ 2.02 signal that also increased after 5 min but, contrary to what was observed in wild type cells, exhibited a clear decrease for longer times (Fig. 2, B and C). The higher intensity of the g ~ 2.02 signal correlates well with the

![Figure 1](Image 60x214 to 288x336)

**FIGURE 1.** Hydrogen peroxide impairs the growth of the E. coli ytfE mutant strain. Shown are E. coli wild type (wt, circles), ΔytfE (ΔytfE, squares), and ΔytfE transformed with pYtfE and expressing ytfE in trans (ΔytfE/pYtfE, triangles), grown in LB under microaerophilic conditions, left untreated (filled symbols) or treated with 2 mM H2O2, at an addition of 2 mM H2O2 caused a very small growth inhibition in the wild type strain, whereas the ΔytfE cells suffered growth impairment, clearly showing that the lack of ytfE results in a strain with higher sensitivity to hydrogen peroxide stress. The ytfE disruption mutant could be complemented by a clone containing the ytfE gene, since the complemented strain grew to final culture densities similar to that of the parent strain following treatment with hydrogen peroxide (Fig. 1).

**TABLE 1**

The E. coli ytfE mutant has a higher concentration of intracellular free iron

| Strain | Percentage of growth compared with untreated control |
|--------|-----------------------------------------------------|
|        | No 2,2′-dipyridyl | 1 mM 2,2′-dipyridyl |
|        | 1 μg/ml streptonigrin | 2 μg/ml streptonigrin | 1 μg/ml streptonigrin | 2 μg/ml streptonigrin |
| Wild type | 81 ± 7 | 58 ± 3 | 95 ± 0.4 | 95 ± 5 |
| ΔytfE | 14 ± 4 | 7 ± 1 | 94 ± 11 | 92 ± 8 |
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FIGURE 2. Comparison of the hydrogen peroxide-induced damage on fumarase A overexpressed in E. coli wild type and ΔytfE cells by EPR and enzyme activity analysis. Wild type (A) and ΔytfE cell extracts (B) were treated with 4 mM H₂O₂, and the EPR spectra were recorded after 1, 5, and 30 min and are presented after subtraction of the EPR spectrum of the wild type untreated cell extracts (A, inset). The unsubtracted EPR spectrum of ΔytfE untreated cells is presented as an inset in B. The EPR intensity scales are the same for A and B. Other EPR parameters are as described under “Experimental Procedures.” C, measured intensities of the EPR signals at g ~ 2.02, attributed to the [3Fe-4S]¹⁺ form of fumarase A, expressed in wild type (filled circles) and ΔytfE (open squares) cell extracts. D, fumarase activity determined in wild type (filled circles) and ΔytfE (open squares) cell extracts treated with 4 mM H₂O₂ immediately after the zero point and normalized for the initial activity of each strain (wild type, 2.7 units/mg protein; ΔytfE, 1.9 units/mg protein).

FIGURE 3. Comparison of the hydrogen peroxide-induced damage on aconitase B overexpressed in E. coli wild type and ΔytfE cells by EPR and enzyme activity analysis. Wild type (A) and ΔytfE (B) cell extracts were treated with 4 mM H₂O₂, and the EPR spectra were recorded after 1, 5, and 30 min and are presented after subtraction of the EPR spectrum of the wild type untreated cell extracts (A, inset). The inset in B shows the unsubtracted EPR spectrum of ΔytfE untreated cells. The EPR intensity scales are the same for A and B. Other EPR parameters are as described under “Experimental Procedures.” C, measured intensities of the EPR signals at g ~ 2.02, attributed to the [3Fe-4S]¹⁺ form of aconitase B, expressed in wild type (filled circles) and ΔytfE (open squares) cell extracts. D, aconitase activity determined in wild type (filled symbols) and ΔytfE (open symbols) treated with 1 mM H₂O₂ (circles) or with 4 mM H₂O₂ (squares) immediately after the zero point and normalized for the initial activity of each strain (wild type, 53.9 milliunits/mg protein; ΔytfE: 25.1 milliunits/mg protein).

The repair of iron-sulfur clusters by YtfE is most probably due to further degradation of the [3Fe-4S]¹⁺ center of aconitase B observed in the wild type cells expressing aconitase B (Fig. 3D). The EPR spectra of cells expressing fumarase A and submitted to NO showed the g ~ 2.04 signal, whose intensity increased with time in the ytfE-depleted cells and remained essentially invariant in the wild type cells (Fig. 4, A–C). Enzymatic data acquired after the first minutes of NO stress showed that a ~40% decrease in the initial activity of fumarase occurred for both strains. However, for longer times, the fumarase activity in the mutant strain exhibited a significant additional decrease, not observed in the wild type (Fig. 4D), indicating that in the ytfE minus background, [Fe-S] clusters are more prone to nitrosative damage.

As expected, the EPR spectra of cells expressing fumarase A and submitted to NO showed the g ~ 2.04 signal, whose intensity increased with time in the ytfE-depleted cells and remained essentially invariant in the wild type cells (Fig. 4, A–C). Enzymatic data acquired after the first minutes of NO stress showed that a ~40% decrease in the initial activity of fumarase occurred for both strains. However, for longer times, the fumarase activity in the mutant strain exhibited a significant additional decrease, not observed in the wild type (Fig. 4D), indicating that in the ytfE minus background, [Fe-S] clusters are more prone to nitrosative damage.

The EPR spectrum of wild type cells expressing aconitase B and exposed for 5 min to NO exhibited a signal with a g value of ~2.02 (Fig. 5A), which can be attributed to the [3Fe-4S]¹⁺ form, since the formation of varying amounts of this species has been faster and stronger decrease of the fumarase activity observed for ΔytfE upon the addition of hydrogen peroxide H₂O₂, when compared with the wild type strain (Fig. 2D). Moreover, the loss of the g ~ 2.02 observed in ytfE mutant cells for longer periods is most probably due to further degradation of the [3Fe-4S]¹⁺ center, as judged by the very low values of fumarase activity in ytfE-depleted cell extracts measured for the same time interval (Fig. 2D).

When submitted to hydrogen peroxide stress, cells producing aconitase B also exhibited the g ~ 2.02 signal, but the E. coli wild type and the ytfE mutant presented different time evolution patterns (Fig. 3, A–C). The intensity of the EPR signal of the [3Fe-4S]¹⁺ center of aconitase B observed in the ytfE mutant cells after hydrogen peroxide exposure was lower than that measured in the wild type cells (Fig. 3, A–C). Enzymatic measurements also revealed that cells expressing aconitase B and treated for 1 min with 4 mM hydrogen peroxide showed a severe loss of aconitase activity, either in wild type or in ΔytfE (Fig. 3D). Hence, the lower intensity of the aconitase g ~ 2.02 signal in ΔytfE is most probably due to a more degraded form of the aconitase [Fe-S] cluster present in the ΔytfE cells that is EPR-silent. Confirmation was obtained by lowering the concentration of hydrogen peroxide to 1 mM, in which case it was observed that the decrease in the aconitase activity caused by oxidative stress was more pronounced in the absence of ytfE (Fig. 3D).

Nitrosative Stress—E. coli fumarase and aconitase produced in wild type and ytfE mutant cells and treated with nitric oxide were also analyzed. Nitrosative stress induced the development of a signal at g ~ 2.04 in the EPR spectrum of fumarase A- and aconitase B-overexpressing cells resulting from the formation of an inactive dinitrosyl-iron-thiol complex, as previously reported (8).
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reported for cytosolic and mitochondrial aconitases treated with NO (8). Interestingly, this form was never observed in the EPR spectra of ytfE mutant cells expressing aconitase B after incubation with NO (Fig. 5B). Analysis of the EPR signal of aconitase B dinitrosyl-iron-thiol complex (g ∼ 2.04) revealed that 30 min after the addition of 150 μM NO, the intensity of the signal was still increasing in ytfE mutant cells and has decreased in wild type cells (Fig. 5C). Since this concentration of NO rapidly induced a very strong loss of aconitase activity in both strains (Fig. 5D), the enzymatic activities were measured at lower NO concentrations (50 μM). Under these conditions, the loss of aconitase activity was enhanced in the ytfE mutant strain compared with the wild type strain (Fig. 5D).

Altogether, these results showed that in the absence of the ytfE gene, hydrogen peroxide or nitrosative stress caused a stronger damage of the [Fe-S] clusters of both aconitase B and fumarase A, which translated into a more rapid loss of the enzymatic activities.

Iron-Sulfur Cluster Repair Is Abolished in the ytfE Mutant—We next addressed the role of ytfE in the repair of [Fe-S] clusters disrupted by nitrosative and oxidative agents. To this end, the reappearance of the activities of cells expressing aconitase or fumarase was assayed in the presence of tetracycline, which blocks protein synthesis and ensures that regain of activity is due only to cluster repair. Moreover, for each enzyme, the stress exposure time was chosen in order to generate an equal decrease of the initial activity in both the wild type and mutant strain.

For cells producing aconitase B and treated with hydrogen peroxide under conditions in which both strains had lost approximately half of the activity, it was observed that, contrary to the wild type strain that could recover full activity, the ytfE mutant was unable to regain any of the lost activity, at least in the period of observation tested (Fig. 6A). A similar behavior was observed for overexpressing aconitase B cells submitted to nitrosative stress (Fig. 6B), showing that the disruption of ytfE completely impaired the recovery of aconitase activity and that ytfE cell extract kept losing activity along the time.

An analogous set of experiments was performed with cells expressing fumarase A and submitted to oxidative or nitrosative stress. After H₂O₂ stress, the wild type strain was able to attain full recovery of the fumarase activity within 10 min if the stress had caused a loss of approximately half of the initial activity (Fig. 7A) and within 1 h in the case of a more prolonged stress (Fig. 7B). On the contrary, no recovery at all of the fumarase activity was observed in the ytfE mutant, indicating that in this strain, the oxidative induced damage was irreversible (Fig. 7, A and B). The damage caused by NO on the fumarase activity was also found to be irreversible in the ytfE mutant, as judged by the absence of any degree of fumarase activity recuperation. In con-
YtfE Is Able to Repair Oxidative and Nitrosative Damaged Iron-Sulfur Clusters—To confirm that the lack of [Fe-S] cluster repair was indeed dependent on the YtfE protein, the same set of experiments was performed for the ytfE mutant strain expressing the iron-sulfur enzymes but this time in the presence of purified E. coli YtfE protein. Hence, to cell lysates of E. coli expressing aconitase B or fumarase A, exposed to hydrogen peroxide or nitric oxide, and scavenged with catalase or hemoglobin, purified holo-YtfE was added. Cells expressing fumarase A treated with 4 mM H$_2$O$_2$ for 1 min (black squares) and for 30 min (gray squares) and expressing aconitase B treated with 1 mM H$_2$O$_2$ for 3 min (open diamonds). B. E. coli ΔytfE cells, treated with tetracycline, were submitted to 4 mM H$_2$O$_2$ for 3 min (A), 1 mM H$_2$O$_2$ for 3 min (B), and to nitrosative stress (4 mM NO for 5 min (black squares) and 10 min (open diamonds)). The stress was stopped by the addition of catalase followed by the addition of 20 μM holo-YtfE protein (black squares), 20 μM apo-YtfE protein (gray circles), 80 μM ferrous iron and subsequent anaerobic incubation (open diamonds), and 20 μM holo-YtfE plus 1 mM 2,2'-dipyridyl protein (asterisks). The activity values are normalized for the initial activity (before) of each strain (ΔytfE expressing fumarase A, 2.5 units/mg protein; ΔytfE expressing aconitase B, 43.3 units/mg protein).

FIGURE 7. A strain lacking ytfE is incapable of repairing oxidative or nitrosative damaged fumarase A. E. coli wild type (filled circles) and ytfE mutant (open squares) cells expressing fumarase A were treated with tetracycline before being subjected to a mild oxidative stress (4 mM H$_2$O$_2$ for 1 min) (A), a prolonged oxidative stress (4 mM H$_2$O$_2$ for 30 min) (B), and to nitrosative stress (150 μM NO for 10 min) (C). The H$_2$O$_2$ and NO exposures were terminated with catalase and hemoglobin, respectively, and enzyme activities were measured immediately after (time 0) and monitored for 1 h afterward. The values are normalized for the initial activity (before) of each strain (wild type, 3.7 units/mg protein; ΔytfE, 2.5 units/mg protein).

FIGURE 6. Repair of aconitase B after oxidative or nitrosative damage is severely impaired in a ytfE mutant strain. E. coli wild type (filled circles) and ytfE mutant (open squares) cell extracts were transformed with pGS783 to express aconitase B, treated with tetracycline, and submitted to 1 mM H$_2$O$_2$ for 3 min (A) or to 50 μM NO for 10 min (B). The H$_2$O$_2$ and NO exposures were terminated with catalase and hemoglobin, respectively, and enzyme activities were measured immediately after (time 0) and monitored for 1 h afterward. The values are normalized for the initial activity (before) of each strain (wild type, 77.9 milliunits/mg protein; ΔytfE, 43.3 milliunits/mg protein).

FIGURE 8. The YtfE protein can repair oxidative and nitrosative damaged fumarase A and aconitase B and lift the ΔytfE phenotype. A, E. coli ΔytfE cells treated with tetracycline were subjected to hydrogen peroxide stress, and immediately after catalase addition, 20 μM purified holo-YtfE protein was added. Cells expressing fumarase A treated with 4 mM H$_2$O$_2$ for 1 min (black squares) and for 30 min (gray squares) and expressing aconitase B treated with 1 mM H$_2$O$_2$ for 3 min (open diamonds). B. E. coli ΔytfE cells, treated with tetracycline, were subjected to nitric oxide stress, and immediately after hemoglobin addition, 20 μM holo-YtfE protein was added. Shown are cells expressing fumarase A treated with 150 μM NO for 5 min (black squares) and cells expressing aconitase B treated with 50 μM NO for 10 min (open diamonds). C. E. coli ΔytfE cells expressing fumarase A, treated with tetracycline, were submitted to 4 mM H$_2$O$_2$ for 1 min. The stress was stopped by the addition of catalase followed by the addition of 20 μM holo-YtfE protein (black squares), 20 μM apo-YtfE protein (gray circles), 80 μM ferrous iron and subsequent anaerobic incubation (open diamonds), and 20 μM holo-YtfE plus 1 mM 2,2'-dipyridyl protein (asterisks). The activity values are normalized for the initial activity (before) of each strain (ΔytfE expressing fumarase A, 2.5 units/mg protein; ΔytfE expressing aconitase B, 43.3 milliunits/mg protein).

Contrast, wild type cell extracts could recover almost all of the initial fumarase activity in a short period of time (Fig. 7C). In summary, these data showed that in the absence of ytfE and under the conditions tested, E. coli is unable to repair [4Fe-4S]$^{2+}$ clusters modified by hydrogen peroxide or nitrosative stress.
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(Fig. 8C). It is worth mentioning that the ytfE mutant did not exhibit auxotrophy for thiamine, a phenotype related to impaired sulfur metabolism (30), and that YtfE has no cysteine desulfurase activity (data not shown). The addition of YtfE to the cell lysates prior to the application of the stress did not prevent the decrease of the enzyme activity observed upon oxidative stress (data not shown), suggesting that YtfE is mainly involved in the repair of damaged clusters and does not protect them from destruction.

DISCUSSION

E. coli ytfE shares transcriptional and phenotypic similarities with isc and suf operons, two systems with important roles in the biosynthesis of iron-sulfur clusters (31–35). Like the suf and isc operons (14–17,36), the ytfE gene is induced by nitric oxide, and the knock-out strain shows higher sensitivity to nitrosative (16) and oxidative stress (this work). The ytfE mutant also exhibits sensitivity to iron starvation (19), which is greater than the one observed for the iscS mutant and lower than that of the sufABCDSE mutant (36). Furthermore, and as also observed for the suf mutant (22), deletion of the ytfE gene led to an increase of intracellular free iron.

Concerning the biogenesis of iron-sulfur clusters in E. coli, it has been reported that disruption of the suf operon yields a strain with lower activity of [Fe-S] cluster-containing enzymes (31, 34). Furthermore, all isc operon-encoded proteins of E. coli seem to be necessary for de novo synthesis and unnecessary for cluster repair, with the exception of IscS (13). In fact, although the iscS mutant is still able to repair the destabilized clusters, the rate at which it occurs is significantly slower than in the wild type cells (13). On the other hand, studies on the Suf system revealed that this system is adapted to sustain [Fe-S] biogenesis under iron starvation and oxidative stress (13, 32, 36). In this work, we show that the activity of two iron-sulfur enzymes, aconitase B and fumarase A, are dependent on YtfE, since their activities in unstressed cell extracts were ~50 and 30% lower in the ytfE mutant strain, respectively. In fact, these results corroborate our previous data, where it was also reported that ytfE is required for full activity of other examined iron-sulfur proteins, whereas enzymes that did not contain iron-sulfur clusters were unaffected by the mutation (19). Furthermore, the results reported here clearly revealed not only that the loss of aconitase and fumarase activities upon oxidation or nitrosylation occurs faster in the ytfE minus background, but also that the absence of ytfE results in a strain incapable of any significant repair, even after periods of time as long as 1 h, contrary to what is observed for the wild type strain that attains full recovery of the enzymatic activities within a few minutes. Only upon the addition of purified holo-YtfE protein to the mutant cell extracts were the fumarase and aconitase activities fully regained and at rates similar to the wild type (Fig. 8).

The sensitivity of aconitase B and fumarase A to oxidative and nitrosative compounds is related with their rapid inactivation upon exposure to these agents, albeit by different mechanisms. Reactive oxygen species like hydrogen peroxide inactivate these enzymes through oxidation of the [4Fe-4S]2+ center, yielding an unstable [4Fe-4S]3+ cluster that spontaneously degrades to a [3Fe-4S]1+ form, with loss of the catalytic iron atom to the bulk solution (28). Nitric oxide reacts with the [4Fe-4S]2+ center, leading to the formation of a catalytically inactive dinitrosyl-iron complex. The repair of the nitric oxide-modified clusters is proposed to require the initial removal of the dinitrosyl-iron complex, followed by reinsertion of ferrous iron and sulfide (37, 38), whereas reactivation of the oxidatively damaged form can be achieved by treatment under reducing conditions with ferrous iron (39). In both cases, the reactivation of the damaged centers involves reinsertion of iron. The fact that the addition of purified holo-YtfE to cell extracts containing iron-sulfur enzymes in the inactive form allows the full recovery of their activity leads us to propose that YtfE is involved in the enzymatic process needed to recruit and integrate the ferrous iron.

In summary, in this study, we have found that the ytfE gene is involved in oxidative stress protection. Furthermore, we have established that YtfE is an important protein for the repair process of [4Fe-4S]2+ clusters damaged by oxidative and nitrosative stress. Further work will be needed to assess the role of YtfE on other types of Fe-S clusters. Nevertheless, it seems that E. coli requires several systems to achieve the assembly and repair of iron-sulfur clusters, and the importance of each system concerning the type of cluster remains to be established.

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REFERENCES

1. Beinert, H., Holm, R. H., and Munck, E. (1997) Science 277, 653–659
2. Johnson, D. C., Dean, D. R., Smith, A. D., and Johnson, M. K. (2005) Annu. Rev. Biochem. 74, 247–281
3. Kiley, P. J., and Beinert, H. (2003) Curr. Opin. Microbiol. 6, 181–185
4. Frazzon, I., and Dean, D. R. (2003) Curr. Opin. Chem. Biol. 7, 166–173
5. Fontecave, M., Choudens, S. O., Py, B., and Barras, F. (2005) J. Biol. Inorg. Chem. 10, 713–721
6. Barras, F., Loiseau, L., and Py, B. (2005) Adv. Microb. Physiol. 50, 41–101
7. Draper, J. C. (1997) Methods 11, 319–329
8. Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997) J. Biol. Chem. 272, 20340–20347
9. Gardner, P. R., Costantino, G., Szabo, C., and Salzman, A. L. (1997) J. Biol. Chem. 272, 25071–25076
10. Rogers, P. A., Eide, L., Klungland, A., and Ding, H. (2003) DNA Repair (Amst.) 2, 809–817
11. Inlay, J. A., and Linn, S. (1988) Science 240, 1302–1309
12. Fliis, D. H., Empate, M. H., and Guest, J. R. (1992) Biochemistry 31, 10331–10337
13. Diaman, O., Outten, F. W., and Inlay, J. A. (2004) J. Biol. Chem. 279, 44590–44599
14. Zheng, M., Wang, X., Templeton, L. J., Smulski, D. R., LaRossa, R. A., and Storz, G. (2001) J. Bacteriol. 183, 4562–4570
15. Lee, J. H., Yeo, W. S., and Roe, J. H. (2004) Mol. Microbiol. 51, 1745–1755
16. Justino, M. C., Vicente, J. B., Teixeira, M., and Saraiva, L. M. (2005) J. Biol. Chem. 280, 2636–2643
17. Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., and Storz, G. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 745–750
18. Johnson, D. C., Unciuleac, M. C., and Dean, D. R. (2006) J. Bacteriol. 188, 7551–7561
19. Justino, M. C., Almeida, C. C., Goncalves, V. L., Teixeira, M., and Saraiva, L. M. (2006) FEMS Microbiol. Lett. 257, 278–284
20. Woods, S. A., Schwartzbach, S. D., and Guest, J. R. (1988) Biochim. Biophys. Acta 912, 71–78
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21. Bradbury, A. J., Gruer, M. J., Radd, K. E., and Guest, J. R. (1996) Microbiology 142, 389–400
22. Nachin, L., El, H. M., Loiseau, L., Expert, D., and Barras, F. (2001) Mol. Microbiol. 39, 960–972
23. Woodmansee, A. N., and Imlay, J. A. (2002) Methods Enzymol. 349, 3–9
24. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
25. Gardner, P. R. (2002) Methods Enzymol. 349, 9–23
26. Massey, V. (1953) Biochem. J. 53, 72–79
27. Bodenmiller, D. M., and Spiro, S. (2006) J. Bacteriol. 188, 874–881
28. Imlay, J. A. (2006) Mol. Microbiol. 59, 1073–1082
29. Han, D., Canali, R., Garcia, J., Aguilera, R., Gallaher, T. K., and Cadenas, E. (2005) Biochemistry 44, 11986–11996
30. Mihara, H., and Esaki, N. (2002) Appl. Microbiol. Biotechnol. 60, 12–23
31. Tokumoto, U., and Takahashi, Y. (2001) J. Biochem. (Tokyo) 130, 63–71
32. Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) EMBO J. 22, 427–437
33. Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) J. Biol. Chem. 278, 38352–38359
34. Schwartz, C. J., Djamam, O., Imlay, J. A., and Kiley, P. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9009–9014
35. Takahashi, Y., and Tokumoto, U. (2002) J. Biol. Chem. 277, 28380–28383
36. Outten, F. W., Djamam, O., and Storz, G. (2004) Mol. Microbiol. 52, 861–872
37. Rogers, P. A., and Ding, H. (2001) J. Biol. Chem. 276, 30980–30986
38. Yang, W., Rogers, P. A., and Ding, H. (2002) J. Biol. Chem. 277, 12868–12873
39. Varghese, S., Tang, Y., and Imlay, J. A. (2003) J. Bacteriol. 185, 221–230