Oxidative stress and disruption of labile iron generate specific auxotrophic requirements in *Salmonella enterica*

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The response of a cell to integrated stresses was investigated using environmental and/or genetic perturbations that disrupted labile iron homeostasis and increased oxidative stress. The effects of the perturbations were monitored as nutritional requirements, and were traced to specific enzymic targets. A *yggX gshA cyaY* mutant strain required exogenous thiamine and methionine for growth. The thiamine requirement, which had previously been linked to the Fe–S cluster proteins ThiH and ThiC, was responsive to oxidative stress and was not directly affected by manipulation of the iron pool. The methionine requirement was associated with the activity of sulfite reductase, an enzyme that appeared responsive to disruption of labile iron homeostasis. The results are incorporated in a model to suggest how the activity of iron-containing enzymes not directly sensitive to oxygen can be decreased by oxidation of the labile iron pool.

INTRODUCTION

Micro-organisms such as *Salmonella enterica* maintain a small pool of labile iron for use in various cellular processes (Petrat *et al.*, 2002). The size of this pool is tightly controlled to ensure sufficient levels of cellular iron for growth while preventing its accumulation, which can be toxic. During aerobic growth, iron can participate in a series of reactions that include the Fenton reaction, resulting in the formation of the highly reactive hydroxyl radical that can damage DNA (equations 1–3) (Keyer & Imlay, 1996; Liochev & Fridovich, 1994; Srinivasan *et al.*, 2000):

Growing evidence has linked a number of cellular factors including YggX, glutathione and CyaY to a role in maintaining labile iron homeostasis and thus preventing the potential toxic effects of labile iron (Ding *et al.*, 2007; Gralnick & Downs, 2001, 2003; Thorgersen & Downs, 2008; Vivas *et al.*, 2006).

Cells lacking YggX exhibit defects consistent with increased oxidative stress and defects in iron homeostasis. These defects have been attributed to a role for YggX in preventing superoxide stress (Thorgersen & Downs, 2008). The connection between YggX and superoxide

occurs at several levels; *yggX* is a member of the Sox regulon (Pomposiello & Demple, 2000), and *yggX* mutant strains are sensitive to the superoxide-generating compound paraquat (Gralnick & Downs, 2001). Strains lacking YggX have an increased GC–TA transversion mutation frequency indicative of Fenton chemistry (Gralnick & Downs, 2003), presumably generated by superoxide (Keyer & Imlay, 1996). Mutations in the *yggX* and *gshA* loci combine to result in decreased activity of Fe–S cluster enzymes and increased sensitivity to reactive oxygen species (Thorgersen & Downs, 2008).

The main cellular reductant, glutathione, is a free thiol that accumulates to millimolar levels in the cell (Helbig *et al.*, 2008; Jocelyn, 1972). Apart from its role as a reductant, glutathione has been shown to chelate metals (Helbig *et al.*, 2008; Li & Manning, 1955; Perrin & Watt, 1971; Sugiura & Tanaka, 1972). Studies have linked glutathione to a role in cobalt resistance (Freeman *et al.*, 2005; Thorgersen & Downs, 2007), and we have proposed that glutathione acts as a chelator for the labile iron pool (Thorgersen & Downs, 2008). Mutants defective at the *gshA* locus are unable to synthesize glutathione and display several phenotypes consistent with a defect in labile iron homeostasis and oxidative stress, including increased expression of Fur-regulated genes, and decreased activity of Fe–S cluster proteins (Gralnick *et al.*, 2000; Thorgersen & Downs, 2008).

CyaY is the prokaryotic homologue of frataxin, a protein that traffics iron in the mitochondria of eukaryotes (Lutz *et al.*, 2001; Puccio & Koenig, 2000; Yoon & Cowan, 2003). Several properties of CyaY support a role for this protein in labile iron trafficking. CyaY has an anionic surface to which

Abbreviations: NDH-1, NADH dehydrogenase complex I; SDH, succinate dehydrogenase.

both Fe^{2+} and Fe^{3+} bind (Nair *et al.*, 2004). The K_d of Fe^{2+} binding to CyaY is approximately 4 μM (Bou-Abdallah *et al.*, 2004). The iron-binding properties of CyaY can be diminished by reducing agents and increased by oxidative stress. This may indicate a role for CyaY in iron homeostasis under conditions of oxidative stress (Ding *et al.*, 2007). Double mutant strains lacking CyaY as well as either YggX or ApbC (a protein involved in Fe-S cluster synthesis) (Skovran & Downs, 2003), have decreased activity of the Fe-S cluster-containing protein NADH dehydrogenase complex I (NDH-1), suggesting that Fe-S cluster metabolism is compromised in these strains (Vivas *et al.*, 2006).

Cobalt can be toxic by competing with iron at various metabolic loci (Thorgersen & Downs, 2007). Exposure of a wild-type *Salmonella* strain to 160 μM cobalt generated a reduced sulfur requirement that was eliminated by iron supplementation but not by anoxic growth (Thorgersen & Downs, 2007). This requirement was traced to a defect in the enzyme sulfite reductase, which contains two iron cofactors, a 4Fe-4S cluster and sirohaem (Christner *et al.*, 1983). The cobalt-dependent effect on sulfite reductase was attributed to competition between iron and cobalt at the enzyme uroporphyrinogen III methylase (CysG), which inserts either iron or cobalt into Factor II to produce either sirohaem or an intermediate in cobalamin synthesis, respectively (Fazzio & Roth, 1996; Goldman & Roth, 1993; Spencer *et al.*, 1993; Stroupe *et al.*, 2003; Thorgersen & Downs, 2007). Thus, CysG was identified as an enzyme

that was sensitive to the manipulation of cellular iron pools. Low sirohaem production by CysG compromises sulfite reductase (CysII) and can generate a nutritional requirement for reduced sulfur (Thorgersen & Downs, 2007).

This study was initiated by identifying nutritional requirements of mutant strains and tracing them to the respective target enzymes. Specifically, sulfite reductase was identified as a target sensitive to oxidative stress via defects in labile iron homeostasis. Supplementing the growth medium with the defined nutritional requirements resulted in restoration of growth to the mutant strains. This supplementation resulted in cells with wild-type growth but decreased overall fitness, which could be uncovered by exposure to an external stress. In-depth analysis of the effects caused by multiple combinations of perturbations to labile iron homeostasis has provided insights into this complex metabolic system.

METHODS

Bacterial strains, media, and chemicals. All strains used in this study are derived from *S. enterica* LT2 and are listed with their respective genotypes in Table 1. Standard genetic techniques were used to construct and verify multiply-mutant strains. The NCE medium of Berkowitz *et al.* (1968) supplemented with 1 mM MgSO_4 was used as minimal medium. Glucose (11 mM) or gluconate (11 mM) was provided as the sole carbon source. Where specified, additional sulfur sources were added to a concentration of 0.3 mM in addition to the 1 mM MgSO_4 already present. Thiamine was used at a

Table 1. Bacterial strains

All strains were part of the lab stock or were generated for this study.

Strain	Genotype*
DM10000	Wild-type
DM9673	<i>yggX::Gm</i>
DM9680	<i>ghsA101::Tn10d(Tc)</i>
DM9684	<i>cyaY::Cm</i>
DM9715	<i>ghsA101::Tn10d(Tc) cyaY::Cm</i>
DM9716	<i>yggX::Gm cyaY::Cm</i>
DM9720	<i>yggX::Gm ghsA101::Tn10d(Tc)</i>
DM9742	<i>yggX::Gm ghsA101::Tn10d(Tc) cyaY::Cm</i>
DM10052	$\Delta\text{ryhB1 acnA}::\text{Kn}$
DM10056	<i>yggX::Gm \Delta\text{ryhB1 acnA}::\text{Kn}</i>
DM10057	<i>ghsA101::Tn10d(Tc) \Delta\text{ryhB1 acnA}::\text{Kn}</i>
DM10062	<i>cyaY::Cm \Delta\text{ryhB1 acnA}::\text{Kn}</i>
DM10063	<i>yggX::Gm ghsA101::Tn10d(Tc) \Delta\text{ryhB1 acnA}::\text{Kn}</i>
DM10064	<i>yggX::Gm cyaY::Cm \Delta\text{ryhB1 acnA}::\text{Kn}</i>
DM10080	<i>ghsA101::Tn10d(Tc) cyaY::Cm \Delta\text{ryhB1 acnA}::\text{Kn}</i>
DM10081	<i>yggX::Gm ghsA101::Tn10d(Tc) cyaY::Cm \Delta\text{ryhB1 acnA}::\text{Kn}</i>
DM10287	<i>cysG1510::Tn10(Tc)</i>
DM11054†	<i>sodA101::Cm sodB111::Kn</i>

*::Gm, ::Cm, ::Kn designations reflect insertion/deletions made by the method of Datsenko & Wanner (2000). Tn10d refers to the transposition-defective mini-Tn10 (Tn10 Δ 16 Δ 17) (Way *et al.*, 1984).

†*sodA101* and *sodB111* alleles were obtained from J. Slauch (University of Illinois at Urbana-Champaign).

concentration of 100 nM. In an effort to control metal concentrations, all minimal media were made using MilliQ-filtered water (MQH₂O) and culture tubes were used a single time. Difco nutrient broth (NB) (8 g l⁻¹) with NaCl (5 g l⁻¹) was used as rich medium, with Difco Biotek agar added to a final concentration of 1.5 % for solid medium. Bismuth sulfite agar was purchased from Becton Dickinson (BD). The growth medium for nitrite reductase assays contained, per litre: 2 g peptone (BD), 7 g KH₂PO₄, 0.05 g NH₄Cl, 0.1 g NaNO₂ and 11 mM glucose. The FeCl₃ and CoCl₂ were added to growth medium after sterilization at indicated amounts from a 0.1 M stock in 0.1 M HCl and a 0.2 M stock in MQH₂O respectively. The final concentrations of antibiotics were as follows: tetracycline, 20 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; gentamicin 6 µg ml⁻¹; and chloramphenicol, 20 µg ml⁻¹. All other chemicals were purchased from Sigma.

Growth analysis. Growth was quantified in liquid medium, using three independent cultures. Strains were grown overnight at 37 °C in NB medium, harvested and resuspended in an equal volume of saline, and 100 µl inoculated into 5 ml of the appropriate medium in 18 × 150 mm culture tubes. Cultures were placed in an air shaker at 37 °C and growth was monitored by following the OD₆₅₀ on a Bausch & Lomb Spectronic 20. The starting OD₆₅₀ was routinely between 0.02 and 0.07 for growth curves.

Cobalt minimal inhibitory concentration (MIC) determination.

Using the growth methods described above, each strain was grown in minimal NCE glucose medium with the indicated additions at several different concentrations of CoCl₂ ranging from 0 to 60 µM. A graph of CoCl₂ concentration versus OD₆₅₀ after 10 h growth was generated for each strain, and the linear portion of the toxicity curve was used to determine a MIC for cobalt. The concentration of CoCl₂ at which the cultures were unable to grow to an OD₆₅₀ of 0.3 within 10 h was defined as the MIC. Reported values represent the mean and standard deviation for three independent cultures.

Sulfide production. Sulfide production was used as an indirect measure of sulfite reductase activity when cobalt was involved. This was necessary since the detection of sulfide in the sulfite reductase assay is inhibited by cobalt. Overnight 2 ml NB cultures were grown at 37 °C. A 2 µl aliquot of the overnight culture was pierced into a bismuth sulfite plate containing 0.3 mM cysteine (Becton Dickinson) prepared with increasing amounts of CoCl₂ (50–250 µM) (Goldman & Roth, 1993). After 12 h incubation at 37 °C the diameter of the black Bi₂S₃ zone surrounding the inoculation site and indicative of sulfide production was measured.

Enzyme assays

Enzyme assays were performed with three independent cultures for each strain. Protein concentrations were determined using the Bradford assay (Bradford, 1976).

Sulfite reductase assays. Overnight 2 ml NB cultures grown at 37 °C were inoculated (200 µl) into 5 ml minimal NCE glucose medium containing 0.15 mM djenkolic acid dihydrochloride as the sole source of sulfur. Cultures were harvested and washed with 1 × NCE minimal salts at an OD₆₅₀ of 0.4–0.6. Cell pellets were resuspended in 300 µl 0.05 M potassium phosphate buffer pH 7.7 and were lysed by sonication. Assays were performed using the method of Dreyfuss & Monty (1963), detecting sulfide production over time. Sulfide was assayed colorimetrically by the method of Siegel (1965). Values are reported as specific activity [µmol sulfide produced min⁻¹ (mg protein)⁻¹].

Nitrite reductase assays. Whole cells were prepared from anaerobically grown cultures for nitrite reductase assays as described previously (Thorgersen & Downs, 2007). Assays used the method of Cole & Ward (1973), detecting nitrite consumption over

time. Nitrite was assayed colorimetrically by the method of Snell & Snell (1949) as modified by Cole & Ward (1973).

Aconitase B (AcnB) assays. Overnight 2 ml NB cultures grown at 37 °C were inoculated (150 µl) into 5 ml minimal NCE gluconate medium supplemented with 0.2 % Casamino acids, 100 nM thiamine and 50 µM FeCl₃ with or without 20 µM CoCl₂. Cell-free extracts were generated, and assays were performed as previously described (Skovran & Downs, 2000). Values are reported as specific activity [ΔA₂₄₀ min⁻¹ (mg protein)⁻¹].

NADH dehydrogenase complex I (NDH-1) assays. NDH-1 activity was assayed as an adaptation from Skovran and Zambrano described by Vivas (Skovran *et al.*, 2004; Vivas *et al.*, 2006; Zambrano & Kolter, 1993). Values are reported as specific activity [ΔA₃₄₀ min⁻¹ mg protein)⁻¹].

Succinate dehydrogenase (SDH) assays. Succinate dehydrogenase activity was assayed in the extracts generated for the AcnB assays above, as described previously (Skovran & Downs, 2000). Values are reported as specific activity [ΔA₆₀₀ min⁻¹ mg protein)⁻¹].

RESULTS

Loss of YggX, GshA and/or CyaY generates a growth requirement for thiamine and/or reduced sulfur

Growth analysis showed that lesions in the *yggX*, *gshA* and *cyaY* loci interact to generate and/or exacerbate nutritional requirements that indicate defects in iron homeostasis. The data in Table 2 show that strains with a lesion in one locus grew with wild-type proficiency in minimal medium. Growth of double mutant strains lacking *gshA* was compromised, with the *yggX gshA* mutant showing no detectable growth on minimal medium. The addition of thiamine restored growth to this strain, as has been previously reported (Gralnick *et al.*, 2000). Growth of the *gshA cyaY* double mutant was enhanced by thiamine, suggesting a similar but less severe cellular defect (data not shown). Introduction of a mutation in the third locus exacerbated the nutritional defect, resulting in a requirement for both thiamine and methionine to achieve full growth.

Growth of the triple mutant strain was tested under numerous conditions to evaluate the effects of oxygen and iron on the growth requirements for thiamine and methionine. Growth in standing cultures, which limits aeration of the culture, eliminated both the thiamine and methionine requirements of the triple mutant strain (Fig. 1a). This contrasted with the sulfur requirement induced by cobalt, which was not corrected by anoxic growth (Thorgersen & Downs, 2007). When 1 mM FeCl₃ was added, growth of the triple mutant strain in minimal glucose medium required thiamine but not methionine (Fig. 1b).

At levels not affecting the wild-type, cobalt potentiated growth defects in strains lacking YggX, GshA and/or CyaY. The data in Table 2 show that, while the wild-type was unaffected, 10 µM cobalt exacerbated the nutritional requirement(s) in the *gshA* mutant and eliminated growth

Table 2. Strains lacking YggX, GshA and CyaY exhibit growth defects in minimal glucose medium with cobalt

Cultures were grown at 37 °C with shaking in minimal NCE glucose (11 mM) medium. The following concentrations were used: thiamine (100 nM), CoCl₂ (10 µM) and methionine (0.3 mM). Values are means ± SD of the specific growth rate of three independent cultures. Specific growth rate (μ) was calculated by the equation $\mu = \ln(X/X_0)/t$, where X is OD₆₅₀, X_0 is OD₆₅₀ at time zero, and t is time (in hours). ND, Not determined; NG, no growth.

Strain	Minimal	Thi	Met	Thi, Met	Co	Co, Thi	Co, Met	Co, Thi, Met
Wild-type	0.25 ± 0.03	ND	ND	ND	0.24 ± 0.03	ND	ND	ND
<i>yggX</i>	0.30 ± 0.03	ND	ND	ND	0.24 ± 0.05	ND	ND	ND
<i>gshA</i>	0.23 ± 0.02	ND	ND	ND	0.12 ± 0.02	0.24 ± 0.05	0.21 ± 0.02	0.25 ± 0.03
<i>cyaY</i>	0.26 ± 0.03	ND	ND	ND	0.25 ± 0.03	ND	ND	ND
<i>yggX gshA</i>	NG	0.16 ± 0.02	NG	0.28 ± 0.02	NG	0.14 ± 0.04	NG	0.20 ± 0.02
<i>yggX cyaY</i>	0.27 ± 0.03	ND	ND	ND	NG	0.20 ± 0.02	0.29 ± 0.02	0.27 ± 0.03
<i>gshA cyaY</i>	0.17 ± 0.03	ND	ND	ND	NG	0.11 ± 0.03	0.16 ± 0.03	0.22 ± 0.02
<i>yggX gshA cyaY</i>	NG	NG	NG	0.27 ± 0.02	NG	NG	NG	0.25 ± 0.03

in each of the multiply-mutant strains. In the majority of strains, the addition of either thiamine or methionine restored significant growth. In both the *yggX gshA* mutant and the triple mutant strain, the addition of cobalt had little or no effect on the growth requirements, suggesting that in these strains the major target(s) of cobalt was damaged by consequences of the mutant background.

The growth medium affects the MIC of cobalt for the mutant strains

The sensitivity of the mutant strains was characterized by defining the MIC of cobalt for the relevant strains in several

Fig. 1. Growth defects in a *yggX gshA cyaY* strain. Wild-type (filled symbols) and *yggX gshA cyaY* (open symbols) strains were grown in minimal glucose medium. (a) Growth conditions were with (squares) or without (circles) aeration. (b) Growth media included no additions (squares), thiamine and iron (diamonds), and methionine and iron (triangles).

media (Table 3). The MIC of cobalt was defined as the concentration of cobalt at which a strain was unable to grow to an OD₆₅₀ of 0.3 within 10 h (see Methods). The data in Table 3 are complementary to the data in Table 2, since they allow conclusions about the fitness, or ability to survive additional perturbation by cobalt, of strains with similar growth rates. Of the single mutant strains, the *gshA* mutant was most sensitive to cobalt. This effect has been reported and it was hypothesized that some of the toxic effects of cobalt are mediated through depletion of the free thiol pool (Thorgersen & Downs, 2007). The ability of the double and triple mutant strains to withstand a cobalt challenge was significantly decreased. Although the growth rate of the triple mutant strain in the presence of 10 µM cobalt was restored to wild-type levels by thiamine and methionine (Table 2), this strain had a lower MIC of cobalt in the presence of both nutrients than did other strains with the same growth rate. One interpretation of this result is that cobalt provides an additional stress on the metabolic network that cannot be adapted to in the absence of one or more of the mutant loci.

The methionine requirement is linked to sulfite reductase activity

Exogenous cobalt affects sulfite reductase activity (and thus methionine synthesis) by decreasing sirohaem synthesis and affecting Fe-S cluster occupancy (Thorgersen & Downs, 2007). Sulfite reductase was assayed to extend our understanding of the interplay between the mutant loci and cobalt (Table 4). Among the single mutants, only the lack of *gshA* affected sulfite reductase activity, decreasing it by ~50 %. Among the double mutants the *yggX gshA* strain showed a defect significantly more severe than the relevant single mutant strains. However, the triple mutant strain again had the most severe defect, with only 15 % of the activity found in the wild-type. When standing conditions were used to decrease aeration of the growing cultures, the

Table 3. Cobalt MICs of mutant strains

Cultures were grown at 37 °C with shaking in minimal NCE glucose (11 mM) medium. The following concentrations were used: thiamine (100 nM) and methionine (0.3 mM). Values are the concentration of CoCl_2 (μM) at which strains were unable to grow above an OD_{650} of 0.3 after 10 h (mean $\pm$ SD of three independent cultures). NG, No growth: cultures failed to reach an OD_{650} of 0.3 in minimal glucose medium with no CoCl_2 .

Strain	Relevant genotype	Minimal	Thi	Met	Thi, Met
DM10000	Wild-type	18.1 ± 0.2	20.4 ± 1.5	38.5 ± 0.8	43.6 ± 1.9
DM9673	<i>yggX</i>	14.9 ± 1.2	19.0 ± 2.8	30.6 ± 3.3	44.8 ± 0.3
DM9680	<i>gshA</i>	4.0 ± 1.7	9.3 ± 1.0	25.1 ± 0.7	36.5 ± 0.2
DM9684	<i>cyaY</i>	17.2 ± 1.3	15.4 ± 1.0	43.1 ± 1.5	41.8 ± 2.5
DM9720	<i>yggX gshA</i>	NG	3.1 ± 3.1	NG	29.3 ± 1.9
DM9716	<i>yggX cyaY</i>	4.0 ± 2.0	8.5 ± 0.7	20.0 ± 5.4	36.0 ± 0.7
DM9715	<i>gshA cyaY</i>	NG	6.1 ± 0.3	13.8 ± 1.1	37.7 ± 1.4
DM9742	<i>yggX gshA cyaY</i>	NG	NG	NG	13.1 ± 1.1

sulfite reductase activity of the wild-type and the triple mutant strain were indistinguishable [5.5 ± 0.1 and $5.6 \pm 0.1 \mu\text{M}$ sulfide produced min^{-1} (mg protein^{-1}) respectively]. Consistently, the activity of another sirohaem-containing enzyme, nitrite reductase, in cells grown under anoxic conditions was no different between the triple mutant strain and wild-type. Thus the mutant effects were negated by lowering the oxygen content of the cultures, which distinguished the mutant effects from those caused solely by cobalt, which were not corrected by anoxic growth conditions (Thorgersen & Downs, 2007).

Bismuth sulfite agar plates were used to assess the sensitivity of sulfite reductase to cobalt in the mutant strains (Fig. 2). The black zone surrounding the inoculation site (indicative of sulfide production) of all the strains was measured in bismuth sulfite plates containing cysteine and increasing concentrations of cobalt after 24 h. Sulfide production in the triple mutant strain was low with no cobalt present, and undetectable with the lowest concentration of cobalt used (50 μM). All strains showed a decrease in sulfide production caused by increasing

cobalt, but in general the strains lacking *cyaY* showed the largest effect by cobalt. In other conditions tested in this study, the loss of CyaY alone did not have a visible effect; however, CyaY appeared to be required for a normal response to a cobalt challenge as measured by sulfide production. This result could indicate that the role of CyaY allows some level of metal specificity that affects the iron-containing cofactors of sulfite reductase, and supports the

Table 4. Sulfite reductase activity of mutant strains

Strain	Relevant genotype	Sulfite reductase activity*
DM10000	Wild-type	7.1 ± 0.9 (1.00)
DM9673	<i>yggX</i>	6.0 ± 1.2 (0.85)
DM9680	<i>gshA</i>	3.2 ± 0.6 (0.46)
DM9684	<i>cyaY</i>	5.7 ± 1.0 (0.81)
DM9720	<i>yggX gshA</i>	1.9 ± 0.3 (0.27)
DM9716	<i>yggX cyaY</i>	5.2 ± 0.9 (0.74)
DM9715	<i>gshA cyaY</i>	2.8 ± 0.5 (0.40)
DM9742	<i>yggX gshA cyaY</i>	1.1 ± 0.4 (0.15)

*Values are specific activity [μmol sulfide produced min^{-1} (mg protein^{-1}) (mean $\pm$ SD)] of three independent cultures. Values in parentheses are relative activity, obtained by dividing the activity of the relevant strain by the activity of the wild-type parent.

Fig. 2. Cobalt decreases sulfide production. Sulfide production was measured indirectly in strains lacking YggX, GshA, CysG or CyaY and/or in the presence of cobalt. A 2 μl sample of an overnight NB culture of the indicated strain was pierced into bismuth sulfite plates supplemented with 0.3 mM cysteine containing increasing amounts of CoCl_2 . The diameter of the black zone radiating from the spot was measured after 24 h of incubation at 37 °C, as an indication of sulfide production.

general model that CyaY is involved in iron trafficking *in vivo*. A *cysG* mutant is unable to make sirohaem. As expected this strain had no sulfite reductase activity and provided a control for any other cellular activities that might produce sulfide.

Superoxide dismutase mutants in *S. enterica*

Labile iron homeostasis and oxidative stress are intertwined, such that disruption of the labile iron pool can result in oxidative stress, and oxidative stress can disrupt the labile iron pool. Therefore, if the triple mutant strain was experiencing oxidative stress, the nutritional requirements of this strain should be replicated by exposing a wild-type strain to oxidative stress. A *S. enterica* strain lacking both cytoplasmic superoxide dismutases (*sodA sodB*) was constructed and its nutritional requirements were assessed. The *sodA sodB* strain failed to grow in minimal glucose medium aerobically, but it grew anaerobically. Supplementation with multiple combinations of amino acids, including those that satisfy the growth requirement of the equivalent *Escherichia coli* strain (Carlioz & Touati, 1986) (branched-chain, sulfur-containing, and aromatic), failed to restore significant growth. NB and minimal glucose supplemented with Casamino acids (0.2%) supported growth of the *sodA sodB* mutant strain with growth rates of 0.23 and 0.32 respectively. However, the mutant had an extended growth lag (3–6 h) compared to wild-type. The *sodA sodB* mutant in our *S. enterica* background appeared to experience an amount of superoxide stress that would prevent the analysis of subtle nutritional requirements, and this mutant was not further characterized herein.

Superoxide can generate similar effects as the loss of YggX, GshA and CyaY

A wild-type *Salmonella* strain was exposed to increasing concentrations of the superoxide-generating compound

paraquat to determine if the hierarchy of nutritional requirements seen with the mutant strains appeared (Fig. 3a). Exposure to 4 μ M paraquat effectively prevented growth of wild-type *S. enterica* in minimal glucose medium. If methionine and thiamine were added to the growth medium, the cells attained significant growth in the presence of 4 μ M paraquat. The effect of thiamine on growth was significant and had previously been noted (Dougherty & Downs, 2006), but methionine alone had only a slight effect.

Sulfite reductase activity was assayed from wild-type cells grown in the presence of increasing concentrations of paraquat (Fig. 3b). As the concentration of paraquat increased, the specific activity of sulfite reductase decreased. At 20 μ M paraquat, the activity of sulfite reductase in a wild-type strain was 48.8% of the activity seen for cells grown without paraquat, suggesting that either directly or indirectly, superoxide stress could damage sulfite reductase. The effect of paraquat on the methionine growth requirement was subtle, indicating that effects of superoxide on sulfite reductase activity were not large enough to generate a growth requirement. This scenario was supported by the relatively weak effect of paraquat on the activity of sulfite reductase *in vitro*. The idea that several enzymes could have significantly lowered activity, but not result in a nutritional requirement, led us to test several different Fe–S cluster proteins that may have distinguishable defects in the triple mutant strain.

Fe–S cluster proteins are compromised in strains lacking YggX, GshA and CyaY

The enzyme sulfite reductase contains a buried 4Fe–4S cluster and sirohaem cofactor, both involved in electron transfer (Christner *et al.*, 1983). The enzyme ThiH, which has previously been linked to the thiamine auxotrophy of a *yggX gshA* mutant strain (Gralnick *et al.*, 2000; Martinez-Gomez *et al.*, 2004), is a member of the SAM radical superfamily of proteins characterized by a solvent-exposed

Fig. 3. Superoxide generates nutritional requirements and decreases sulfite reductase activity. (a) Wild-type *S. enterica* was grown in the presence of increasing concentrations of paraquat (PQ) in defined medium with (filled symbols) or without (open symbols) methionine and with (circles) or without (squares) thiamine. Thus, cultures represented by open squares contained no supplements and those with filled circles contained both. Growth is reported as a percentage of the growth in the absence of paraquat after 24 h incubation. (b) Specific activity of sulfite reductase in cell-free extracts of wild-type cells grown in defined medium with increasing concentrations of paraquat.

Table 5. Strains lacking YggX, GshA and CyaY have decreased activity of Fe–S cluster enzymes

Strain	Relevant genotype*	AcnB activity†	SDH activity†	NDH-1 activity†
DM10052	Wild-type	3950 ± 331 (1.00)	2850 ± 254 (1.00)	4470 ± 327 (1.00)
DM10056	<i>yggX</i>	2780 ± 241 (0.70)	2150 ± 148 (0.75)	3460 ± 289 (0.78)
DM10057	<i>gshA</i>	3020 ± 158 (0.76)	1620 ± 421 (0.57)	2810 ± 167 (0.63)
DM10062	<i>cyaY</i>	2660 ± 212 (0.67)	2100 ± 245 (0.74)	4090 ± 379 (0.92)
DM10063	<i>yggX gshA</i>	1510 ± 111 (0.38)	550 ± 154 (0.19)	970 ± 223 (0.22)
DM10064	<i>yggX cyaY</i>	2100 ± 113 (0.53)	1400 ± 76 (0.49)	2060 ± 204 (0.46)
DM10080	<i>gshA cyaY</i>	1790 ± 193 (0.45)	1160 ± 131 (0.41)	1990 ± 300 (0.45)
DM10081	<i>yggX gshA cyaY</i>	1640 ± 192 (0.41)	690 ± 287 (0.24)	320 ± 163 (0.07)

*All strains lack *ryhB acnA*.

†Values are specific activity [$\Delta A \text{ min}^{-1} (\text{mg protein})^{-1}$ (mean $\pm$ SD)] of three independent cultures. Values in parentheses are relative activity, obtained by dividing the activity of the relevant strain by the activity of the *ryhB acnA* parent.

oxygen-labile 4Fe–4S cluster (Berkovitch *et al.*, 2004; Kriek *et al.*, 2007; Layer *et al.*, 2003). The activities of three other Fe–S cluster-containing proteins with different properties were assayed in the mutant strain backgrounds to understand the effects that these mutations have on Fe–S cluster/iron metabolism. Significantly, each enzyme seemed to be affected differently.

Aconitase B (AcnB) is a member of the dehydratase family of proteins, characterized by a solvent-exposed 4Fe–4S cluster involved in substrate binding (Gardner & Fridovich, 1991). These clusters are known to be sensitive to multiple forms of oxidative stress (Flint *et al.*, 1993; Jang & Imlay, 2007). AcnB was assayed in a *ryhB acnA* background to negate regulatory effects of Fur in the mutant strains, and eliminate activity of the oxygen-stable AcnA protein (Masse & Gottesman, 2002; Thorgeresen & Downs, 2007). Western hybridization experiments determined that similar levels of AcnB protein were present in all strains. The effects on AcnB activity in the mutant backgrounds were different from those seen for sulfite reductase (Table 5). The *gshA* single mutant was not greatly decreased in activity compared to the other single mutant strains, and the relative aconitase activity in the triple mutant strain compared to wild-type was significantly higher (41 % as opposed to 15 %) than with other enzymes.

The NADH dehydrogenase complex 1 (NDH-1), encoded by the *nuo* genes, contains nine Fe–S clusters and is part of the electron-transport chain (Friedrich & Bottcher, 2004; Hinchliffe & Sazanov, 2005). Succinate dehydrogenase (SDH) contains three Fe–S clusters (Yankovskaya *et al.*, 2003). Both SDH and NDH-1 contain buried Fe–S clusters involved in electron transfer, like sulfite reductase. Unlike sulfite reductase, SDH and NDH-1 do not contain sirohaem. The activities of SDH and NDH-1, like sulfite reductase, were lowest in the *gshA* mutant strain as compared to the other single mutant strains. Significantly, only NDH-1 had lower activity in the triple mutant strain as compared to the *yggX gshA* strain, indicating that CyaY may play a significant role in the

maturation of this complex when other components of the system are compromised.

DISCUSSION

Labile iron homeostasis and oxidative stress can be viewed as a cycle. Perturbations that increase labile iron result in oxidative stress through Fenton chemistry. Likewise, oxidative stress damages oxygen-labile Fe–S cluster proteins, increasing intracellular labile iron levels, resulting in Fenton chemistry (Keyer & Imlay, 1996). A *yggX gshA cyaY* triple mutant strain displays phenotypes, including nutritional requirements and decreased activity of iron-containing enzymes, indicating that this strain experiences increased oxidative stress and has defects in iron homeostasis.

Requirements for thiamine and methionine are generated by distinct mechanisms

The nutritional requirements of the triple mutant strain (for thiamine and methionine) result from distinct targets that display different sensitivities to oxidative stress and disruption of the iron pool. The thiamine requirement appears to be directly caused by oxidative stress, and the methionine requirement appears to be caused by disruption of iron homeostasis, which can be caused indirectly by oxidative stress. In support of this model, both requirements in the triple mutant strain can be suppressed by anoxic growth (Fig. 1a), but iron supplementation specifically suppressed the methionine requirement and not the thiamine requirement (Fig. 1b). Also, cobalt can disrupt iron homeostasis in the absence of oxygen, and was shown to cause a requirement for reduced sulfur but not thiamine in the absence of oxygen (Thorgeresen & Downs, 2007). Further, cobalt generated a thiamine requirement only in strains lacking either YggX or GshA, which have increased oxidative stress (Gralnick & Downs, 2001; Thorgeresen & Downs, 2008). Consistently, the superoxide-generating compound paraquat caused a strong

thiamine requirement but only a slight demand for increased methionine (Fig. 3a).

The enzyme targets that are the cause of the nutritional requirements have properties consistent with the above hypothesis. The thiamine auxotrophy in a *yggX gshA* strain was previously observed and was linked to the 4Fe–4S cluster proteins ThiH and ThiC (Dougherty & Downs, 2006; Gralnick *et al.*, 2000; Martinez-Gomez *et al.*, 2004). Both ThiH and ThiC belong to the family of SAM radical proteins that contain a solvent-exposed oxygen-labile Fe–S cluster and have no activity when assayed in the presence of oxygen (Kriek *et al.*, 2007; Leonardi & Roach, 2004; Martinez-Gomez & Downs, 2008). Proteins in this family are known to be sensitive to superoxide (Berkovitch *et al.*, 2004; Layer *et al.*, 2003). The methionine requirement was traced to sulfite reductase, which contains an Fe–S cluster buried within the structure of the protein (Christner *et al.*, 1983). This enzyme retains activity when assayed in the presence of oxygen (Dreyfuss & Monty, 1963).

Insights into the roles of YggX, glutathione and CyaY in iron homeostasis

Each of the single and multiple mutant strains disrupted in *yggX*, *gshA* and/or *cyaY* displayed differences in nutritional requirements, sensitivity to cobalt, and enzyme activities that provide insights into the role of these components in cellular metabolism. Of the single mutant strains, the strain that cannot synthesize glutathione (*gshA*) displayed the most severe defects, including sensitivity to cobalt and decreased activity in all the Fe–S cluster enzymes tested with the exception of AcnB. Loss of GshA sensitized the strain so that cobalt targeted both the methionine and thiamine nutritional requirements (Table 3). Our current model suggests that glutathione participates as a labile iron chelator in the cell. In this scenario, the loss of glutathione would increase the pool of iron available for Fenton chemistry (Thorgersen & Downs, 2008). Such a role for glutathione would position it at the intersection of the labile iron pool and oxidative stress. Such a central role could explain the strong phenotypic consequences of a *gshA* mutation seen in this and other studies.

YggX has been proposed to affect labile iron homeostasis through an interaction with superoxide (Thorgersen & Downs, 2008). In this study, the *yggX* mutant strain was sensitive to cobalt in a thiamine-specific manner. Our hypothesis that the thiamine requirement is directly linked with oxidative stress is consistent with the proposed model for YggX and this observation.

The defects associated with the *cyaY* mutant strain were subtle and consisted of a slight sensitivity to cobalt and decreased sulfite reductase activity in the presence of cobalt. Despite the apparently small impact, disruption of *cyaY* had significant effects in combination with *gshA* and/or *yggX* mutations, emphasizing the complexity of the integrated system. In the absence of CyaY, sulfide

production was highly sensitive to cobalt. This result suggests that the presence of CyaY aided in metal selectivity, an observation consistent with the current model of CyaY as a labile-iron trafficking protein.

Other Fe–S cluster protein targets of direct/indirect oxidative stress

The observation that several of the mutant strains remained sensitive to cobalt even when their nutritional requirements were satisfied indicated that these strains were less fit and suggested that additional targets were compromised in the mutant backgrounds. Consistently, the activities of the Fe–S cluster enzymes tested, AcnB, SDH and NDH-1, were decreased in several of the mutant strains. Of these enzymes, AcnB retained the most activity in the mutant backgrounds. This result was unexpected since AcnB is a member of the dehydratase family of proteins and is known to contain a solvent-exposed 4Fe–4S cluster that is oxygen labile (Gardner & Fridovich, 1992).

The enzymes sulfite reductase, SDH and NDH-1 have Fe–S clusters that are involved in electron transfer and that are buried within the protein (Christner *et al.*, 1983; Friedrich & Bottcher, 2004; Hinchliffe & Sazanov, 2005; Yankovskaya *et al.*, 2003). These enzymes retain activity in the presence of oxygen yet their activities in the mutant strains were significantly decreased. We propose that these enzymes are targets sensitive specifically to defects in labile iron homeostasis and that the observed sensitivity of these enzymes to oxidative stress is indirect, occurring via the labile iron pool. This conclusion is supported by the above discussion linking the methionine requirement to a disruption in iron homeostasis rather than oxidative stress, since the methionine requirement was traced to the enzyme sulfite reductase. Sulfite reductase, SDH and NDH-1 also share the characteristic that their activities are lower in the *gshA* strain as compared to the other single mutant strains.

The study of integrated metabolic systems can be complicated by the existence of indirect effects. Iron homeostasis in particular is difficult to study due to its connection to oxidative stress through Fenton chemistry. Studying multiple targets affected by oxidative stress and perturbations to iron homeostasis in a *yggX gshA cyaY* strain has provided insights that will facilitate the dissection of connections linking iron homeostasis to the rest of the metabolic network.

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