Cobalt Targets Multiple Metabolic Processes in *Salmonella enterica*[▽]

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Cobalt is essential for growth of *Salmonella enterica* and other organisms, yet this metal can be toxic when present in excess. Wild-type *Salmonella* exhibits several metabolic defects when grown in the presence of cobalt, some of which generate visible growth consequences. Work herein identifies sulfur assimilation, iron homeostasis, and Fe-S cluster metabolism as targets for cobalt toxicity. In each case it is proposed that cobalt exerts its effect by one of two mechanisms: direct competition with iron or indirectly through a mechanism that involves the status of reduced thiols in the cell. Cobalt toxicity results in decreased siroheme production, increased expression of the Fur regulon, and decreased activity of Fe-S cluster proteins. The consequences of reduced sulfite reductase activity in particular are exacerbated by the need for glutathione in cobalt resistance. Significantly, independent metabolic perturbations could be detected at cobalt concentrations below those required to generate a detectable growth defect.

Trace metals are necessary for the growth of living organisms, partly because they are cofactors for several essential enzymes. The requirement for these metals is complicated by the fact that they can be toxic at elevated concentrations. Heavy metal toxicity has been described over the years in a variety of organisms. Despite the long-standing recognition of metal toxicity, the mechanism(s) of toxicity is not completely understood. The toxicities of several metals, including iron, copper, cadmium, and nickel, involve interactions with oxygen that result in radical generation (reviewed in reference 45). Cobalt in particular has been shown to (i) generate a spectrum of reactive oxygen species in water (25) and (ii) result in free radicals in circulating rat blood in the presence of ascorbic acid (50). Further, *Escherichia coli* strains lacking both cytoplasmic superoxide dismutases were found to be more sensitive to cobalt than wild-type strains (16).

Other reports have suggested cobalt toxicity is generated by a competition with iron, with which it shares several similarities in atomic properties. For instance, iron-enriched medium decreased induction of the erythropoietin gene by cobalt in human Hep3B cells (20). In *Saccharomyces cerevisiae*, Aft1 is an iron-binding transcriptional activator of genes involved in iron uptake and homeostasis. Cobalt was found to induce genes that were part of the Aft1 regulon. Significantly, this cobalt-induced phenotype was suppressed by the addition of iron to the growth medium (44). In *E. coli*, *rcnA*, which encodes a transporter involved in cobalt and nickel efflux (37), was found to be regulated by the ferric uptake regulator (Fur) (23). Finally, in *Salmonella enterica*, strains lacking *yggX* and one of several other genes (*apbC*, *apbE*, *gshA*, and *rseC*) implicated in Fe-S cluster metabolism were shown to have a cobalt-induced thiamine auxotrophy which could be suppressed by iron (41). In the last case it was hypothesized that cobalt could compete

with iron in the process of Fe-S cluster synthesis and/or repair of the ThiH enzyme (41).

The enzyme uroporphyrinogen III methylase (CysG) directly connects the metabolism of cobalt and iron. CysG inserts iron into factor II to make the cofactor siroheme or cobalt into factor II to make an intermediate in the synthesis of cobalamin (13, 17, 43, 46). Thus, CysG serves as a site where cobalt and iron naturally “compete,” presumably in a manner that is regulated based on the relevant environmental conditions. Two cellular enzymes, sulfite reductase (8) and nitrite reductase (9, 27, 31, 32), require siroheme. This further connects the metabolism of cobalt and iron with that of sulfur and nitrogen.

In *S. enterica*, intracellular iron levels are controlled primarily by the action of Fur. Fur is a transcriptional repressor that is active when bound to ferrous iron (reviewed in references 6 and 18). In iron-replete cells, Fur represses a number of genes involved in iron uptake, including the gene *entB* (47; reviewed in reference 36). Fur indirectly increases the expression of several genes that either use or store iron, including aconitase B (AcnB) and succinate dehydrogenase (SDH), via the RyhB small RNA, whose expression is controlled by Fur (28, 29).

This study was initiated to better understand the target(s) and mechanism(s) of cobalt toxicity in *S. enterica*, particularly as they relate to iron metabolism. A physiological approach was taken to identify the first nutritional requirement generated by growth in cobalt. Subsequently, other metabolic defects were probed at the molecular level, leading to the identification of multiple targets and overlapping mechanisms with distinct properties of cobalt toxicity in *S. enterica*. The results herein determined that multiple metabolic processes are disrupted by cobalt prior to the manifestation of a growth defect and served to emphasize the subtle interactions and metabolic complexity that can complicate interpretation of apparently simple nutritional defects.

MATERIALS AND 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

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TABLE 1. Bacterial strains

Strain ^a	Genotype
DM10000.....	Wild type
DM7623.....	<i>fpr::MudJ zxx-8077::Tn10d</i> (Cm)
DM7879.....	<i>entB103::MudJ zxx-8077::Tn10d</i> (Cm)
DM7881.....	<i>entB103::MudJ ghsA101::Tn10d</i> (Tc) <i>zxx-8077::Tn10d</i> (Cm)
DM7885.....	<i>entB103::MudJ fur-1 zbj-5123::Tn10</i> (Tc) ^b
DM8792.....	<i>ryhB1::Cm fur-1 zbj-5123::Tn10</i> (Tc)
DM10052.....	<i>ΔryhB1 acnA::Kn</i>
DM10287.....	<i>cysG1510::Tn10</i> (Tc)

^a All strains were part of the lab stock or were generated for this study.
^b *zbj-5123::Tn10*(Tc) is 33% linked by cotransduction to the *fur-1* allele.

Table 1. All strains were shown to express YggX by Western blot analyses. The NCE medium of Berkowitz et al. (4) supplemented with 1 mM MgCl₂ was used as minimal medium. Glucose (11 mM), gluconate (11 mM), or succinate (16.5 mM) was provided as the sole carbon source. Na₂SO₄ (1 mM) served as the sole source of sulfur unless otherwise specified. Alternative sulfur sources glutathione (1 mM), cysteine (0.3 mM), Na₂SO₃ (1 mM), and Na₂S (0.2 mM) were used as indicated. Cysteine and sulfide were used at lower concentrations due to their toxic effects on growth, and concentrations that were chosen allowed optimal growth of the wild-type strain in minimal glucose medium. In an effort to control metal concentrations, all minimal media were made using Milli-Q filtered water (MQH₂O), and culture tubes were used a single time. Luria broth (LB) and Difco nutrient broth (NB; 8 g/liter) with NaCl (5 g/liter) were used as rich media, with Difco BifTek agar added to a final concentration of 1.5% for solid media. Chelexed LB for β-galactosidase assays was made with MQH₂O and incubated/stirred for 1 h with 1 g/liter Chelex 100 resin (Sigma Chemical Co., St. Louis, MO) prior to filtration and sterilization. Bismuth sulfite agar was purchased from Becton Dickinson and Co. (BD), Sparks, MD. The growth medium for nitrite reductase assays contained, per liter, 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 the indicated amounts from a 0.1 M stock in 0.1 N HCl and a 0.2 M stock in MQH₂O, respectively. The final concentrations of antibiotics were as follows: tetracycline, 20 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Growth analysis. Growth was quantified in liquid medium, monitoring three independent cultures for each strain under each condition. Strains were grown overnight at 37°C in NB medium, harvested, and resuspended in an equal volume of saline, and 100 μl was inoculated into 5 ml of the appropriate medium in 18-by 150-mm culture tubes. Cultures were placed in an air shaker at 37°C, and growth was monitored by measuring the optical density at 650 nm (OD₆₅₀) on a Bausch & Lomb Spectronic 20. The starting OD₆₅₀ was routinely between 0.02 and 0.07 for growth curves. Plots are arithmetic to emphasize the lag phase, which was a significant aspect of the observed phenotype.

Strain construction. Standard genetic techniques were used to construct and verify multiply mutant strains. The *ryhB* deletion/insertion mutant containing the selectable chloramphenicol cassette was constructed by the λ-red recombination method (12). The *ryhB* deletion was constructed from the *ryhB* deletion/insertion mutant containing the selectable chloramphenicol cassette by the λ-red recombination method (12).

Sulfide production. Sulfide production was measured using bismuth sulfite plates supplemented with 0.3 mM cysteine (Becton Dickinson, Sparks, MD) (17). Overnight 2-ml NB cultures were grown at 37°C. Two microliters of the overnight culture was stabbed into the center of a bismuth sulfite plate prepared with increasing amounts of CoCl₂ (50 to 350 μM). After a 12-h incubation at 37°C, the diameter of the black Bi₂S₃ zone surrounding the inoculation site was measured.

Enzyme assays. All enzyme assays were performed using three independent cultures for each strain. Protein concentrations were determined using the Bradford assay (5).

(i) **Nitrite reductase assays.** Overnight 5-ml NB cultures were inoculated into 125-ml Erlenmeyer flasks filled with 120 ml medium. Cells were grown at 37°C without shaking for 24 h before harvesting. Cells were washed with 10 ml 50 mM phosphate, pH 7.4, and were resuspended in a final volume of 1 ml of wash buffer. Assays were performed using the method of Cole (10), detecting nitrite consumption over time. Nitrite was assayed colorimetrically by the method of Snell and Snell as modified by Cole (10, 42). Activity is reported as μmol nitrite reduced/OD₆₅₀/min.

(ii) **β-Galactosidase assays.** Overnight 2-ml NB cultures grown at 37°C were inoculated (50 μl) into 5 ml of chelexed LB with added MgSO₄ (1 mM), trace minerals (1×) lacking iron (3), and FeCl₃ (50 μM). Culture tubes were placed in an air shaker at 37°C, and cultures were grown to an OD₆₅₀ of 0.4 before harvest and resuspension in 14.6 mM NaCl. Cells were assayed for β-galactosidase activity according to the method of Miller (30).

(iii) **Aconitase assays.** Overnight 2-ml NB cultures grown at 37°C were inoculated (150 μl) into 5 ml minimal NCE gluconate medium with cysteine as the sole sulfur source and supplemented with 50 μM FeCl₃ and the indicated amounts of CoCl₂. Cell extracts were generated, and assays were performed as previously described (40).

(iv) **Succinate dehydrogenase assays.** Succinate dehydrogenase activity was assayed in the extracts generated for the aconitase assays above. Assays were performed as described previously (40).

(v) **Malate dehydrogenase assays.** Malate dehydrogenase activity was assayed in the extracts generated for the aconitase assays above. Assays were performed using an adaptation of a method used by Cooper (11). The 200-μl reaction mixture consisted of 3 mM NAD⁺, 100 mM DL-malic acid, and 20 to 40 μg of protein in 100 mM Tris, pH 7.4. The rate of the reaction was measured as the change in absorbance at 340 nm over time. Reaction rates were normalized to protein concentration.

RESULTS

A wild-type strain of *S. enterica* showed no significant growth defect in rich medium (LB) with concentrations of cobalt as high as 500 μM (data not shown). In minimal glucose medium the same strain failed to grow in the presence of 160 μM cobalt after 16 h. In the absence of cobalt, growth of this strain was complete by 12 h (Fig. 1). Partial suppression of the cobalt-induced growth defect, which was primarily a growth lag, was seen by altering the composition of the medium with respect to iron or sulfur. Growth with standing cultures to generate anoxic conditions did not suppress the growth defect (data not shown). Titration experiments determined that addition of 1 mM FeCl₃ allowed the strain to grow to full density within 10 h (Fig. 1A), while 250 μM FeCl₃ restored full growth within 24 h (data not shown). In both cases, the addition of iron restored

FIG. 1. Exogenous FeCl₃ and sulfur source affect growth in the presence of CoCl₂. Growth of wild-type *Salmonella enterica* LT2 on minimal glucose medium with (filled symbols) and without (open symbols) 160 μM CoCl₂ was monitored over time as the A₆₅₀. (A) Cultures were grown with Na₂SO₄ as a sole sulfur source in the absence (squares) or presence (circles) of FeCl₃ (1 mM). (B) Cultures were grown in medium with different sole sulfur sources as follows: Na₂SO₄ (1 mM; squares), Na₂SO₃ (1 mM; triangles), Na₂S (0.2 mM; circles), cysteine (0.3 mM; diamonds). Plots are arithmetic to emphasize the lag phase, which was a significant aspect of the observed phenotype.

FIG. 2. The sulfur assimilation pathway. Shown is a schematic representation of the sulfur assimilation pathway and its connection to reactions catalyzed by the enzyme CysG. The enzyme complex CysIJ requires a 4Fe-4S cluster and the cofactor siroheme. Gene products catalyzing various reactions are indicated by the relevant arrows.

a growth rate similar to that of the no-cobalt control but did not entirely eliminate the lag before exponential growth began.

An observation made during routine growth analysis suggested an effect of sulfur source on growth in the presence of cobalt. In addition to sulfate, provided in the standard medium, wild-type *S. enterica* can utilize other compounds as the sole sulfur source (e.g., sulfite, sulfide, and cysteine). The growth profile (i.e., rate and final density) of the wild-type strain on media with any of these compounds as sulfur sources was not significantly different (data not shown). However, in medium with cobalt (160 μ M), the sulfur sources could be ordered by their ability to decrease the growth lag (Fig. 1B) as follows: sulfate, sulfite, sulfide, and cysteine. This order paralleled that of the sulfur assimilation pathway, in which inorganic sulfate is sequentially reduced by several enzymatic steps, culminating in the formation of cysteine (Fig. 2). These data suggested that cobalt caused a partial block in the pathway for sulfur assimilation, preventing growth on minimal medium.

Sulfite and nitrite reductase activities are compromised by growth with cobalt. Considering the impact of iron described above, the sulfite reductase enzyme (CysIJ) stood out as a potential target for cobalt toxicity in the sulfur assimilatory pathway due to both its 4Fe-4S cluster and siroheme cofactors. A model was previously proposed in which competition between iron and cobalt could impact the occupancy status of the 4Fe-4S cluster of ThiH (41), and a similar scenario was considered for CysIJ. Further, competition between iron and cobalt could lead to bias in the siroheme/cobalamin intermediate ratio when excess cobalt was present. This latter scenario was attractive, since other studies had determined that the specificity of uroporphyrinogen III methylase (CysG), which incorporates either iron or cobalt into factor II to generate siroheme or an intermediate in cobalamin synthesis, could be manipulated (13).

A working model suggested cobalt caused a partial block in sulfur assimilation by decreasing 4Fe-4S cluster integrity

FIG. 3. Sulfide produced by wild-type *Salmonella enterica* is decreased by cobalt. A 2- μ l sample of an overnight NB culture of wild-type *Salmonella enterica* was injected into bismuth sulfite plates supplemented with 0.3 mM cysteine containing increasing amounts of CoCl_2 . The diameter of the black zone surrounding the spot, indicative of sulfide production, was measured after 24 h of incubation at 37°C. (A) Representative plates with indicated CoCl_2 concentrations. (B) Plot of zone diameter measured at each CoCl_2 concentration in triplicate.

and/or decreasing siroheme production. A simple prediction of this model was that both sulfite reductase and nitrite reductase would have decreased activity in cells grown in high levels of cobalt. Sulfite reductase can be measured indirectly in vivo by monitoring sulfide production. When cells are stabbed into the surface of a bismuth sulfite plate, a black precipitate that is proportional to the excreted sulfide forms (17). As shown in Fig. 3, the diameter of the black precipitate zone generated by a wild-type strain was inversely proportional to the concentration of cobalt added to bismuth sulfite plates containing cysteine. As predicted, a *cysG* mutant failed to generate a detectable precipitate on similar plates (data not shown). Control experiments showed that these cobalt concentrations did not affect growth when measured in liquid medium with the same nutrient composition as the bismuth sulfite plates. This result was consistent with the hypothesis that the defect leading to growth inhibition in minimal medium by cobalt was decreased CysIJ activity. However, these data did not differentiate between a defect in one or both of the relevant cofactors for this enzyme.

Nitrite reductase was assayed in whole cells by monitoring the disappearance of nitrite from an assay mixture. Cells were grown in complex medium with or without 160 μ M cobalt anoxically in the presence of nitrite in order to allow expression of the *nirB* gene, whose product (nitrite reductase) requires the cofactor siroheme. Data in Fig. 4 show the relative nitrite reductase activities of wild-type cells grown with or without 160 μ M cobalt. In the absence of cobalt, nitrite reduction in the wild type showed a linear increase over time. When the cells were grown in the presence of cobalt, no significant nitrite reduction was detected up to 180 min. In fact, the activity in the wild-type strain grown in cobalt was about the same as that of a *CysG* mutant which cannot produce siroheme (Fig. 4). Addition of 1 mM FeCl_3 to the medium restored nitrite reductase activity to that found in the absence of cobalt.

Lack of glutathione exacerbates cobalt sensitivity. The effect of cobalt on sulfur assimilation appeared to be more complex than a partial block at CysIJ. A single target for cobalt in the

FIG. 4. Nitrite reductase activity is decreased in cells grown in the presence of CoCl_2 . The disappearance of nitrite over time was measured in assay mixtures containing whole cells grown anoxically in the presence of NaNO_2 as described in Materials and Methods. The wild-type strain (filled symbols) and a *cysG* mutant (open symbols) were grown in the absence (circles) or presence of added metals, 160 μM CoCl_2 (squares) or 160 μM CoCl_2 and 1 mM FeCl_3 (triangles).

sulfur assimilation pathway would be expected to result in two classes of sulfur metabolites, those before the block (and thus unable to enter the pathway) and those after. Consistently, a strain lacking *cysG* is unable to use sulfate or sulfite as a sulfur source and exhibits full growth without lag when sulfide or cysteine is provided (data not shown). The observed gradient effect of sulfur source on the cobalt-dependent growth lag suggested a more complex scenario. First, when cobalt was present, sulfite allowed better growth than sulfate as a sole source of sulfur (Fig. 1). Though both enter the sulfur assimilation pathway before the putative block, various scenarios of flux and enzyme kinetics could explain the differences in their behavior. The more telling result was that sulfide and cysteine (both downstream of the proposed block) were comparable in restoring growth to a *cysG* mutant but not a wild-type strain growing in cobalt. This result indicated that cobalt toxicity was not simply due to indirectly compromising CysIJ. Serine acetyltransferase (CysE) has been shown to be the limiting step in cysteine and glutathione synthesis in various organisms (19), suggesting that addition of sulfide might generate only a limiting amount of cysteine. In such a scenario, if accumulation of a metabolite downstream of cysteine were involved in cobalt resistance, the observed difference of growth on sulfide and cysteine would be expected. These results, and a previous study implicating glutathione in metal resistance (14), led to a hypothesized role for glutathione in cobalt resistance in *Salmonella*. In growth studies, a *gshA* mutant was more sensitive to cobalt than a wild-type strain, as shown in Fig. 5. These data showed that cysteine was not sufficient to eliminate the growth defect caused by cobalt in a *gshA* mutant. Since this strain is unable to convert the cysteine to glutathione (Fig. 2), it suggested that glutathione was needed for full resistance to cobalt toxicity.

The Fur regulon is affected by cobalt levels. A scenario in which competition with iron at CysIJ contributes to cobalt

FIG. 5. Glutathione is required for cysteine-mediated cobalt resistance. Final optical densities at 650 nm of strains grown in various concentrations of cobalt are shown. Results are shown for wild-type (squares) and *gshA* (circles) strains in minimal glucose B1 medium containing either 1 mM Na_2SO_4 (open symbols) or 0.3 mM cysteine (filled symbols) as a sole source of sulfur. Data were gathered after 14 h of growth at 37°C.

toxicity predicted that additional targets for this competition might exist. Fur was such a potential target. An *entB::lacZ* gene fusion serves as a reporter for Fur-mediated repression (47; reviewed in reference 36). Strain DM7879 (*entB::lacZ*) was grown in chelexed LB with increasing concentrations of cobalt, and transcription of the *entB* gene was measured using β -galactosidase assays. Data in Fig. 6 show that *entB* transcription increased proportionally with the cobalt in the medium, consistent with iron and cobalt competing for the iron-binding site of Fur. Such a competition would be consistent with a report that Fur could bind cobalt in vitro (1). At a concentration of 80 μM cobalt, the transcription of *entB* approached that of a

FIG. 6. The presence of CoCl_2 increases expression of the Fur-regulated gene *entB*. (A) Cells containing an *entB-lacZ* gene fusion, DM7879, were grown in chelexed LB growth medium with added trace minerals, 50 μM FeCl_3 , and the indicated concentration of CoCl_2 to an OD_{650} of 0.4 before harvesting and assaying for β -galactosidase activity. (B) β -Galactosidase activity from cells of strain DM7879 grown in the presence of 80 μM CoCl_2 with the following additions: 1 mM FeCl_3 (lane 1), 0.3 mM cysteine (lane 2), or 1 mM glutathione (lane 3). Lane 4 shows β -galactosidase activity from cells of strain DM7879 grown in the presence of 80 μM CoCl_2 under standing (i.e., anoxic) culture conditions. Lane 5 shows transcription of the *entB-lacZ* fusion in a *fur* mutant strain, DM7885.

FIG. 7. Growth on succinate is more sensitive to cobalt than growth on glucose. Growth of the DM8792 (*fur rhyB*) strain, in minimal medium containing 0.3 mM cysteine as the sole sulfur source and 50 μM exogenous FeCl₃, was monitored over time as the OD₆₅₀. Growth was tested in the absence (open symbols) and presence (filled symbols) of 80 μM CoCl₂ with either glucose (squares) or succinate (circles) as the sole carbon source.

strain lacking *fur*, analogous to no iron binding. Titration of FeCl₃ into the medium with 80 μM CoCl₂ resulted in increasing repression of *entB* transcription, showing that the Fur protein had not been damaged by the presence of cobalt. The cobalt-dependent increase in *entB* expression could also be eliminated by anoxic growth or the addition of cysteine or glutathione to the growth medium. Growth of strain DM7879 in 2 mM deferrioxamine (to limit available intracellular iron by chelation) had the expected result of increasing the expression of *entB* (data not shown). However, this increase was not affected by the addition of cysteine or glutathione and was affected only minimally by anoxic growth (data not shown). This latter result suggested cobalt was doing more than simply competing with iron for occupancy of Fur and supported a role for glutathione levels in determining cobalt toxicity. Further, these results identified a cellular component affected by cobalt distinct from enzymes in the sulfur assimilation pathway. It was worth noting that Fur activity was significantly impacted at cobalt concentrations well below the level needed to generate a detectable growth defect.

Cobalt decreases the activity of Fe-S-containing proteins.

The above results showed that addition of cobalt disrupted the Fur regulon and thus resulted in physiological consequences not directly caused by cobalt. Therefore, to probe the metabolic consequence(s) of cobalt on the tricarboxylic acid (TCA) cycle enzymes AcnB and SDH (see below), Fur-mediated transcriptional effects on the relevant genes had to be eliminated. This was accomplished by removing the small regulatory RNA *rhyB*, which is responsible for the Fur-mediated regulation of several genes relevant to this study (29). A *fur* mutant strain also lacking *rhyB* was generated. Growth of strain DM8792 (*fur rhyB*) was monitored on medium with glucose or succinate provided as the sole carbon source.

When strain DM8792 (*fur rhyB*) was grown with glucose as a carbon source and cysteine as a sulfur source, up to 80 μM cobalt had no effect on growth (Fig. 7). When succinate was

FIG. 8. Fe-S cluster proteins are less active in the presence of cobalt. Cells of strain DM10052 (*acnA rhyB*) were grown in minimal gluconate medium containing 0.3 mM cysteine as the sole sulfur source and 50 μM exogenous FeCl₃ with the indicated concentrations of CoCl₂. Cultures were grown with shaking (filled symbols). Cell extracts were generated from each of three cultures under each condition, and the activities of aconitase (circles), succinate dehydrogenase (squares), and malate dehydrogenase (triangles) were measured. Open symbols represent the activity of aconitase from strains grown under standing (i.e., anoxic) culture conditions.

used as the sole carbon source, overall growth was slower, as expected, and the strain failed to grow in the presence of 80 μM cobalt. This experiment suggested that cobalt was compromising the TCA cycle, which is required for growth on succinate. The addition of up to 1 mM FeCl₃ to the succinate medium failed to improve the growth of the strain in the presence of cobalt (data not shown), distinguishing the cobalt toxicity in this situation from that impacting sulfur assimilation.

Three TCA cycle enzymes were assayed for activity: AcnB and SDH, which contain Fe-S clusters, and malate dehydrogenase (MDH), which does not contain an Fe-S cluster. In an *acnA rhyB* double mutant strain (DM10052), any aconitase activity measured would be due to AcnB, which has a labile Fe-S cluster (48). Strain DM10052 was grown in minimal gluconate medium containing 50 μM exogenous iron with various concentrations of cobalt. Results presented in Fig. 8 show that activities of AcnB and SDH were inversely proportional to the cobalt concentration in the medium. Activity of MDH from the same extracts showed no dependence on cobalt concentration. As anticipated by the lack of *rhyB*, Western analyses showed that similar amounts of AcnB accumulated over the entire range of cobalt concentrations (data not shown). These data allowed the conclusion that the cobalt in the medium specifically affected the Fe-S cluster enzymes. Addition of 1 mM exogenous FeCl₃ to the growth medium (with 150 μM cobalt) failed to restore AcnB activity or SDH activity; however, a slight (<2-fold) increase in activity was seen if 1 mM glutathione was added to cultures grown in the presence of cobalt at up to 300 μM cobalt (data not shown). Extracts generated from cells grown in standing cultures to generate anoxic conditions showed a less dramatic decrease in aconitase activity due to cobalt than extracts grown with oxygen present.

DISCUSSION

This study was initiated to identify the metabolic basis of cobalt toxicity in *S. enterica*. During the review of this work a study on cobalt toxicity in *E. coli* was published (35). The authors of that study concluded that cobalt affected the [Fe-S] cluster assembly process during synthesis or repair, a hypothesis we had put forward in a previous study (41). The data presented herein further support this hypothesis and identify additional intertwined metabolic targets of cobalt toxicity: sulfur assimilation, iron homeostasis, and Fe-S cluster metabolism. The data show that the toxicity of cobalt directed at the sulfur assimilation pathway can be eliminated by the addition of excess iron. In contrast, iron had little effect on the toxicity of cobalt directed at Fe-S cluster metabolism, which could be reversed by anoxic growth conditions. The toxicity of cobalt directed at iron homeostasis appeared to be most complex, including components suppressed by either iron or anoxic growth. To account for the in vivo and in vitro results presented, a model involving two distinct but intertwined mechanisms by which cobalt is toxic is suggested. One mechanism involves direct competition with iron, and the other invokes an ability of cobalt to affect iron homeostasis in the cell by oxidizing free thiols.

Cobalt targets sulfur assimilation by direct competition with iron at CysG. The studies presented here suggest that the assimilatory pathway for sulfur is at the center of cobalt toxicity in *S. enterica*. This pathway includes an enzyme with two iron-containing cofactors and is also necessary to produce glutathione, which has been shown here and elsewhere to be involved in cobalt resistance (14). The sulfur-specific growth phenotypes caused by cobalt could be corrected by additional iron, suggesting a competition between the two transition metals was key to this effect. The probable location for this competition is the CysG enzyme, the final enzyme required for siroheme synthesis. A similar competition between cobalt and iron was suggested for a heme protein involved in erythropoietin stimulation (20). Although both siroheme-containing enzymes also contain a 4Fe-4S cluster, the primary effect of cobalt on sulfite and nitrite reductase was concluded to be due to compromised siroheme production. This conclusion was based on the response of these enzymes to iron and anoxic growth conditions, compared to the response of other proteins with only Fe-S cluster targets.

Cobalt compromises the function of Fe-S cluster proteins. The enzymes SDH and AcnB of the TCA cycle were used to monitor the effect of cobalt on Fe-S cluster proteins. Aconitase B and SDH retained ~40% activity at cobalt concentrations as high as 300 μ M. This was in contrast to the siroheme-containing proteins, which had no detectable activity when grown in the presence of 160 μ M cobalt. Also, unlike the defect traced to siroheme deficiency, iron was unable to correct the Fe-S cluster defect in SDH and AcnB caused by cobalt. The inability of iron to restore activity to these proteins suggests either that metal competition does not play a significant role in cobalt toxicity at Fe-S clusters or that the damage to Fe-S clusters by cobalt is irreversible. Reduced synthesis of glutathione, or its depletion (see below), could also contribute to decreased activity, since glutathione has been shown to be involved in Fe-S cluster repair (15). Additional work will be required to dissect

the molecular mechanism(s) by which cobalt impacts Fe-S cluster enzymes. These studies will be facilitated by continuing progress on the multiple proteins that are involved in the formation and repair of Fe-S clusters (22, 24, 26, 39, 41, 49).

Cobalt affects iron homeostasis via the endogenous thiol pool. Growth in the presence of cobalt resulted in derepression of the Fur-regulated gene *entB*, a phenotype that could be reversed by the addition of iron, cysteine, or glutathione to the growth medium or by anoxic growth. A simple scenario suggests that cobalt competes with iron at the Fur iron-binding site. However, this model alone does not account for the effects of cysteine and glutathione on *entB* expression in the presence of cobalt. Significantly, when *entB* expression is increased with an iron chelator, neither cysteine nor glutathione is able to restore repression. Also, although Fur has been shown to bind cobalt in vivo, the Fur-cobalt complex retains the ability to bind DNA and presumably act as a repressor (1). We suggest that cobalt-mediated depletion of glutathione pools is responsible for some of the effects noted here. In this scenario, processes dependent on labile iron, such as Fur iron binding and Fe-S cluster metabolism, could be perturbed by disrupting the intracellular thiol pools. The Fur protein senses labile iron (2) that is chelated to various compounds in the cytoplasm, including thiols (reviewed in reference 34). Cobalt has been shown to oxidize glutathione in vitro (reviewed in reference 7) and disrupts both Fur activity and the activity of Fe-S cluster proteins in a manner that is at least partially suppressed by the addition of cysteine or glutathione to the growth medium. Consistent with this scenario, a *gshA* mutant that cannot synthesize glutathione has increased *entB* transcription (data not shown) and was previously shown to have slower Fe-S cluster repair rates compared to the wild type (15). The suppression of certain cobalt-dependent phenotypes by anoxic growth may suggest that thiols are necessary to maintain iron homeostasis primarily when oxygen is present.

Derepression of the Fur regulon provides a mechanism of cobalt resistance. Derepression of the Fur regulon in *S. enterica* by cobalt shows similarities to the reported induction of the iron regulon by cobalt in yeast (44). The mechanism of iron regulation is distinct in these two organisms (51; reviewed in references 6 and 18), yet addition of cobalt led to the induction of iron uptake genes and iron corrected cobalt toxicity in both cases. Induction of Fur-regulated genes may protect *Salmonella* (and other organisms) from cobalt toxicity by increasing the concentration of intracellular iron, thus out-competing cobalt. This model is supported by the finding that the cobalt efflux protein, RcnA, is a part of the Fur regulon (23).

Results from our lab have suggested links between iron homeostasis and cobalt toxicity in various mutant strains compromised in Fe-S cluster metabolism (41). Preliminary results show these mutants are both more sensitive to cobalt than a wild-type strain and have additional cobalt-dependent defects. The study presented here sought to increase our understanding of the layers at which cobalt affects the physiology and metabolism of a wild-type *Salmonella* strain. Based on this work, we are now in a better position to use cobalt-related phenotypes as a useful tool in dissecting the role of accessory proteins in iron homeostasis and Fe-S cluster metabolism.

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Although not directly cited in the article, a number of studies on iron homeostasis and oxidative stress from the Imlay lab have been instrumental in our thinking (21, 33, 38) and provided an important context for this work.

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