Sulfite Reduction in Mycobacteria

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Abstract

*Mycobacterium tuberculosis* places an enormous burden on the welfare of humanity. Its ability to grow and its pathogenicity are linked to sulfur metabolism, which is considered a fertile area for the development of antibiotics particularly because many of the sulfur-acquisition steps in the bacterium are not found in the host. Sulfite reduction is one such mycobacterial-specific step, and is the central focus of this paper. Sulfite reduction in *Mycobacterium smegmatis* was investigated using a combination of deletion mutagenesis, metabolite screening, complementation and enzymology. The initial-rate parameters for the purified sulfite reductase from *M. tuberculosis* were determined under strict anaerobic condition (\(k_{cat} = 1.0 \pm 0.1\) electrons consumed per second, and \(K_m(SO_3^-) = 27 \pm 1\) \(\mu\)M), and the enzyme exhibits no detectible turnover of nitrite - which need not be the case in the sulfite/nitrite-reductase family. Deletion of sulfite reductase (*sirA*, originally misannotated *nirA*) reveals that it is essential for growth on sulfate or sulfite as sole sulfur sources, and, further, that the nitrite reducing activities of the cell are incapable of reducing sulfite at a rate sufficient to allow growth. Like their nitrite reductase counterparts, sulfite reductases require a siroheme cofactor for catalysis. *Rv2393* (renamed *che1*) resides in the sulfur-reduction operon, and is shown for the first time to encode a ferrocheletase – catalysts that insert \(Fe^{2+}\) into siroheme. Deletion of *che1* causes cells to grow slowly on metabolites that require sulfite reductase activity. This slow-growth phenotype was ameliorated by optimizing growth conditions for nitrite assimilation, suggesting that the nitrogen and sulfur assimilation overlap at the point of ferrocheletase synthesis and delivery.
Introduction

The human genome does not encode the sulfur reduction and cysteine biosynthetic enzymes found in many pathogenic bacteria. The species specificity of these enzymes and the essential metabolic nature of sulfur recommend them as potential targets for antimicrobial development. A considerable literature links sulfur metabolism to the pathogenicity and antibiotic susceptibility of *M. tuberculosis*. Specific sulfolipids correlate well with the virulence of *M. tuberculosis* (1-5) and are reported to inhibit phagosome-lysosome fusion (6), which is critical to survival of the bacterium in the macrophage (7, 8). A more recent literature calls into question whether the segregation of sulfolipid across virulent and avirulent strains is a manifestation, rather than a root cause, of virulence (9, 10), and efforts to trace this lineage toward its root are underway (11, 12). Mycothiol, the mycobacterial equivalent of glutathione, utilizes a cysteine thiol to provide the antioxidant protection the organism needs to survive, particularly during oxidative stress (13), and lower mycothiol levels correlate with enhanced susceptibility to antibiotics, including rifampacin and isoniazid (14). The cysteine biosynthetic pathway, a primary means of assimilating sulfur, has been linked to survival of the organism during the chronic phase of infection, the basis of which may lie in the resistance to reactive oxygen and nitrogen species (15). The current work explores the reduction of sulfite, an essential step in the biosynthesis of cysteine, in the model organism, *Mycobacterium smegmatis*.

Mycobacterial assimilation of sulfate begins with its active transport into the cell, whereupon it is chemically activated by the enzyme ATP sulfurylase (*cysDN*), to form activated sulfate (APS, adenosine 5’-phosphosulfate), Figure 1. APS is then either phosphorylated, by APS kinase (*cysC*), to form the universal sulfuryl-group donor, PAPS (3’-phosphoadenosine 5’-phosphosulfate), or reduced, by APS reductase (*cysH*), to form sulfite (20, 21). If sulfate assimilates through the sulfuryl-transfer branch of the
pathway, the sulfuryl-group is transferred from PAPS, via sulfotransferases, to metabolic recipients whose activities are regulated by the modification. If, on-the-other-hand, sulfate is drawn into the reductive branch of the pathway, by the action of APS reductase (22, 23), the resulting sulfite is reduced further, in a six-electron reduction, to sulfide by the enzyme sulfite reductase (sirA). Sulfide is then incorporated into cysteine, by O-acetyl-L-serine sulphydrylase (cysK1) (24), and from there the sulfur atom, originally present in sulfate, flows into a myriad of reduced-sulfur containing metabolites.

Nitrite and sulfite reductases share a number of similarities including a conserved catalytic architecture designed to guide an essential siroheme cofactor to function as the active center for a six-electron reduction of substrate to product (21, 25, 26). These enzymes are sufficiently similar that either will often catalyze the reduction of both sulfite and nitrite (21), suggesting that sulfur and nitrogen metabolism may be redundant at the point of sulfite/nitrite reduction, and/or in the provision of the siroheme cofactor. Such redundancy is particularly pertinent in the case of M. tuberculosis which must persist amid high levels of destructive reactive-nitrogen species, which it elicits from activated T-cells during infection (27).

The genes encoding APS reductase (cysH) and sulfite reductase (sirA) are located in the sulfur reduction operon (see below), in both M. smegmatis and M. tuberculosis, along with a third coding region, Rv2393, that encodes a protein of unknown function. In the current study, the sulfite reduction step in M. smegmatis was explored by deletion mutagenesis, metabolite screening, enzymatic characterization and complementation.
Materials and Methods

Bacterial strains and cultures. The bacterial strains used in this study are listed in Table 1. The *E. coli* XL1-blue and HB101 strains, used for cloning, and BL21 (DE3) Rosetta pLysS strain, used for expression, were propagated in Luria–Bertani (LB) broth or on LB agar at 37 °C. *M. smegmatis* mc²155 strain was grown at 37 °C in 7H9 liquid medium (Difco) supplemented with 0.2 % glycerol (vol/vol), 0.2 % glucose and 0.05 % Tween 80, or Middlebrook 7H10 solid medium supplemented as described above. L-Cysteine was obtained from the Sigma Chemical Co. The following antibiotics were used at the concentrations indicated: ampicillin (100 µg/mL) (Fisherbiotech), kanamycin A (25 µg/mL) (Sigma), hygromycin B (50 µg/mL, *E. coli*; 50-150 µg/mL, *M. smegmatis*) (Roche).

To obtain the growth curves associated with Fig 4, *M. smegmatis* was grown to late exponential phase at 37 °C, washed 6–8 times in M9 minimal salts without a sulfur source [Na₂HPO₄ (42 mM), KH₂PO₄ (24 mM), NaCl (9.0 mM), NH₄Cl (19 mM), glucose (0.5% wt/vol), tween 80 (0.05% v/v)], and suspended in pre-warmed M9 medium supplemented with a specific source of sulfur, SO₄²⁻ (1.0 mM), Na₂S (1.0 mM), Na₂SO₃ (1.0 mM) or cysteine (200 µM), and cultured further by shaking at 37 °C. Bacterial growth was monitored at 600 nm. To normalize the wild-type and mutant genetic backgrounds in the strains used in Figure 4C, the wild-type strain was transformed with pMV361, which carries h₄₅ and integrates into the genome (31), and pMV261 which confers kanamycin resistance. The mutant strains associated with Figure 4C were transformed using derivatives of pMV261 that contained either the wild type sirA (pRP15) or Rv2393 (pRP16).

The cell-growth studies presented in Figure 6 were accomplished by shaking cells overnight at 37 °C in
the following M9 minimal medium: NaNO$_3$ (20 mM), cysteine (200 μM), Na$_2$HPO$_4$ (42 mM), KH$_2$PO$_4$ (24 mM), NaCl (9.0 mM), glucose (0.5% wt/vol), tween 80 (0.05% v/v). The cells were then washed, as describe, above in pre-warmed M9 medium lacking a sulfur source. The growth studies were initiated by suspending the washed cells in prewarmed M9 medium with either cysteine or sulfate.

**Ferrocheletase complementation.** To assess whether \( \text{Rv2393} \) encodes a ferrocheletase, an \( \text{E. coli} \) cysteine auxotroph that requires ferrocheletase function for growth on \( \text{SO}_4^{2-} \) (strain 302Δa: a \( \text{cysG} \) deletion strain containing pER247 - a P15-origin plasmid that expresses uroporphrinogen III methyltransferase) was transformed with ColE1-origin plasmids that express Rv2393 either from \( \text{M. tuberculosis} \) (pRP17) or \( \text{M. smegmatis} \) (pRP18) and then tested for the ability to grow on \( \text{SO}_4^{2-} \) as a sole source of sulfur. Conversion to prototrophy was assessed on minimal agar containing either \( \text{SO}_4^{2-} \) or cysteine as the sulfur source. Minimal agar contained: Na$_2$HPO$_4$ (42 mM), KH$_2$PO$_4$ (24 mM), NaCl (9.0 mM), NH$_4$Cl (19 mM), agar (15 g/L), CaCl$_2$ (0.10 mM), MgCl$_2$ (1.0 mM), glucose (2.0 %), and either MgSO$_4$ (2.0 mM) or cysteine (280 μM). 302Δa carrying pKK (which expresses \( \text{E. coli} \) CysG) was used as the positive control for growth; the negative growth-control strain was 302Δa carrying pER247 and pET23a (the empty \( \text{Rv2393} \) expression vector). All plates were incubated at 37 °C for 24 to 48hrs.

**DNA manipulation.** Restriction enzymes (REs) were purchased from New England Biolabs and digestions were performed according to manufacturer’s recommendations. The purification of DNA from agarose gels and the isolation of plasmid DNA were done using QIAquick Gel Extraction and QIAprep Spin Miniprep Kits (Qiagen) according to the manufacturer’s protocols. Isolation of \( \text{M. tuberculosis} \) and \( \text{M. smegmatis} \) chromosomal DNA was carried out as described previously (32). Standard heat-shock protocols were used for transformation of \( \text{E. coli} \) strains (33).
**Plasmid Construction.** All of the plasmids and primers used in this study are listed in Tables 2 and 3, respectively. *The SirA expression vector.* Expression of catalytically competent SirA requires co-expression of CysG, which produces the quantities of the siroheme cofactor needed for stoichiometric incorporation into SirA (36). The construction of the co-expression plasmid, pJR2, was accomplished in two steps. First, a 1.7 kb fragment containing sirA was PCR amplified from *M. tuberculosis* genomic DNA using primers TBsirA F and TBsirA R, and inserted into the NdeI and NotI sites of pSKB4 (35), yielding pJR1. In the second step, *E. coli* cysG was PCR amplified (primers colicysG F and colicysG R) and cloned into pJR1 linearized with NotI and BsaAI. The resulting plasmid, pJR2, was used to transform *E. coli* BL21 (DE3) Rosetta pLysS strain for expression and purification (see below). This strain was named JR01. *The complementation plasmids.* *M. smegmatis* sirA and Rv2393 were PCR amplified from genomic DNA using primer pairs smegsirA F/smegsirA R and smegRv2393 F/smegRv2393 R, respectively. In both cases, the forward and reverse primers introduced *Pvu*II and *Hind*III restriction sites, respectively, that were used to subclone the PCR products into pMV261 (cleaved with the same enzymes), producing plasmids pRP15 (sirA) and pRP16 (Rv2393). pMV261 contains the mycobacterial *hsp60* promoter which facilitates constitutive expression of *M. smegmatis* SirA and *M. smegmatis* Rv2393 (31). All constructs were sequenced (AECOM DNA sequencing facility) to confirm the fidelity of the clones.

**Construction of *M. smegmatis* gene-deletion mutants.** The *M. smegmatis* gene-deletion mutants were constructed in three stages, preparation of recombinant cosmids containing the DNA sequences needed for allelic exchange (allelic exchange substrates, AESs), obtaining the high-titer mycobacteriophages needed to isolate genomic deletion events (which occur at low levels), and transduction and selection for allelic exchange. The protocols used to create mycobacterial gene deletion mutants have been described.
previously (32).

**Cosmid construction.** DNA flanking the 5′- and 3′- regions of *sirA* was PCR amplified, using primer pairs 1/1’ and 2/2’ (Figure 3A, and Table 3). The flanking regions were subcloned directionally, using the Van911 restriction enzyme, on either side of the hygromycin-resistance/sacB gene-cassette found in cosmid p0004S. The resulting recombinant cosmid, pMS2391, contained the AES needed to construct the bacteriophage. Using an identical protocol, primer pairs 3/3’ and 4/4’ were used to generate pMS2393, which contains the *Rv2393* AES.

**Mycobacteriophage construction.** The AES-cosmids and purified phagemid DNA (phAE159) were digested separately with *PacI*, ligated together, and *in-vitro* packaged (using the GIGApackIII Gold Packaging Extract, Stratagene) to produce the transducing λ-bacteriophage. *E. coli* HB101 was then transduced with the phage, and transductants were selected on media containing hygromycin (150 μg/mL). Phasmid construction was confirmed by *PacI* digestion prior to electroporation of the phasmid DNA into *M. smegmatis*. Electroporated cells were plated on 7H10 media, and incubated at the permissive temperature, 30 °C, for 3 days. All transducing phages were plaque-purified and high titre phage lysate (10¹⁰ to 10¹¹ plaque forming units/mL) was prepared. *M. smegmatis* was then transduced by mixing late-log phase cells with phage lysate (1:1 v/v ratio) overnight at 37 °C. Transductants were plated onto 7H10 plates containing hygromycin (75 μg/mL) and cysteine (40 μg/mL), and incubated at the non-permissive temperature, 37 °C, for 5 days. *M. smegmatis* deletion mutants were screened by Southern blot analysis (see below).

**Southern blot.** Five μg of *M. smegmatis* genomic DNA was digested completely with either the *BanII* or
**Expression and purification of the M. tuberculosis SirA.** LB media was inoculated with an over night culture of JR01 at an A_600_ of 0.01 and the cells were grown at 37 °C until the density reached an OD600 of ~ 0.7, at which point SirA expression was induced by adding isopropyl-1-thio-D-galactopyranoside (IPTG) to a final concentration of 0.80 mM. The incubation temperature was then shifted to 17°C and cells were harvested by centrifugation 17 hours later. The cell pellet was suspended in 4.2 mL/g cell paste of lysis buffer (KPO_4_ (50 mM, pH 8.0), KCl (0.4 M), PMSF (290 μM), Pepstatin A (1.5 μM), lysozyme (0.10 mg/mL)), and the solution was stirred for 1 hour at 4 °C prior to sonication on ice (Branson Sonifier). Cellular debris was removed by centrifugation (20 min at 31 000 g) and the supernatant was loaded onto a 5 mL Chelating Sepharose Fast flow column charged with Ni^{2+} and equilibrated with Buffer A (KPO_4_ (50 mM, pH 7.3), KCl (0.4 M)). The column was washed with 10 bed volumes of Buffer B (KPO_4_ (50 mM, pH 7.3), KCl (0.4 M), imidazole (10 mM)). The His-GST tagged fusion protein was then eluted from the Ni^{2+}-chelated Sepharose column with 10 bed volumes of Buffer C (KPO_4_ (50 mM, pH 7.3), KCl (0.4 M), imidazole (250 mM), KCl (0.40 M)). The N-terminal His-GST tag was removed by digestion with Prescission Protease (GE Healthcare) during overnight dialysis against Hepes/K^+ (25 mM,
pH 7.5), KCl (100 mM), glycerol (5 %) and β-mercaptoethanol (5.0 mM) at 4 °C. To remove the tag and any uncut fusion protein, the dialyzed, proteolysate was passed back over the Ni$^{2+}$-affinity column and washed extensively with buffer B to remove the SirA, which exhibited an affinity for the resin. Typically, 10 mg of protein (> 95% pure, as judged by eye from Coomasie stained SDS-PAGE gels) was obtained per liter of culture.

**Sulfite reductase assay.** *M. tuberculosis* SirA was assayed for sulfite and nitrite reductase activity under anaerobic conditions activity using methyl viologen (MV) as an electron donor, which was reduced with Zn metal immediately prior to use (37, 38). The assay mix contained O-acetyl-serine (OAS, 5.0 mM), sodium sulfite (13 μM), reduced methyl viologen (250 μM), O-acetyl-serine sulfhydrylase (OASS, 1.5 μM) and SirA (1.3 μM), 50 mM Hepes/K$^+$ (pH 8.0), T = 25 (± 3) °C. The assay was carried out under anaerobic conditions: argon gas scrubbed with reduced MV and ascorbic acid was used to de-gas all buffers and to maintain all samples under positive pressure in a vacuum manifold. A custom optical cuvette that allowed maintenance of positive pressure while stirring was used to optically monitor reactions. The assay was initiated by the addition of the sodium salts of sulfite or nitrite. The enzymatic consumption of either sulfite or nitrite was monitored by following the decrease in absorbance at 684 nm ($\varepsilon = 4.8 \times 10^3$ M$^{-1}$ cm$^{-1}$) caused by the oxidation of MV (39).
Results

The sulfur reduction operon – an overview. The *M. smegmatis* and *M. tuberculosis* sulfur reduction operons are quite similar (Fig. 1A). At their 5'-termini, they begin with *sirA* (sulfite reductase) followed by *cysH* (APS reductase) and *Rv2393* (a hypothetical protein). In both organisms, each of the three coding regions appear to be translationally coupled (i.e., the stop codon of the 5'-gene overlaps the start codon of its downstream partner). The homologous coding regions in the two strains are similar in both length and sequence (Table 1, Fig. 2A) (40). The metabolically linked, sulfate transport operons, which include an ABC transporter (encoded by *cysTWA*) and a sulfate binding protein (encoded by *subI*), are antiparallel to the sulfur reduction operon in both cases, but differ in that they are separated by ~ 5 kb in *M. tuberculosis* and only 10 bp in *M. smegmatis*.

Construction of *sirA* and *Rv2393* deletion mutants. To begin to assess the role of *sirA* and *Rv2393* in-*vivo*, each gene in *M. smegmatis* was deleted using allelic exchange. First, allelic exchange cosmids containing a selectable gene cassette (*γδ res hyg sacB res γδ*) flanked by ~ 900 bp regions of DNA that spanned the 3'- and 5'-edges of *sirA* or *Rv2393* were constructed (see Material and Methods). The allelic exchange substrates were then incorporated into the conditionally replicating phasmid phAE159, in-*vivo* packaged into λ phage heads, and transduced into *E. coli* HB101. Recombinant shuttle cosmids were purified from Hyg<sup>R</sup> *E. coli* transductants and converted into mycobacteriophage-packaged DNA molecules by transfecting them into *M. smegmatis* cells. These cells were plated for phage plaques at the permissive temperature of 30 °C. High-titre transducing lysates were obtained by propagation of the mycobacteriophage in *M. smegmatis*. The lysates were used to transduce *M. smegmatis* at the non-permissive temperature (37 °C) and plated on selective media. Restriction of phage replication results in a
double crossover event between the homologous DNA arms flanking the disrupted gene (32). Fifteen transductants were obtained in the case of sirA, six in the case of Rv2393. Six isolates from each set were tested by Southern blot analysis, all of which had undergone the expected allelic replacement. A single representative from each set is shown in Figure 3B. The observed shifts in DNA fragment size caused by insertion of the Hyg$^R$-SacB gene cassette into the genomic copies of sirA and Rv2393 are in excellent agreement with the expected values – 1.4 and 1.3 kb, respectively.

**sirA is essential for growth in minimal media.** Upon entering the macrophage, *M. tuberculosis* maintains expression of SirA, a sulfite reductase that catalyzes the 6-electron reduction of sulfite (41). Frequently, both sulfite and nitrite can be reduced by either nitrite or sulfite reductases, which are classified on the basis of their substrate preference (21). Thus, a strain lacking sirA might obtain the sulfide needed for cysteine biosynthesis from the reduction of sulfite by other reductases, which would render sirA non-essential, and perhaps diminish its efficacy as antimicrobial target. To establish whether the organism depends essentially entirely on SirA for the provision of sulfide, the *M. smegmatis* sirA-deletion mutant was tested for its ability to grow, relative to wild-type, on sulfur metabolites that straddle either side of the point-of-action of sirA in the cysteine biosynthetic pathway. When fed downstream metabolites (i.e., $S^2$ or cysteine), the mutant and wild-type growth rates are virtually indistinguishable; however, when fed the upstream metabolites ($SO_4^{2-}$ or $SO_3^{2-}$), the mutant shows no detectible growth over a 50 hr incubation, while the wild-type grew with a doubling time of 7.1 (± 0.4) hrs (see Figure 4A). *sirA* is clearly essential for growth on $SO_4^{2-}$ or $SO_3^{2-}$, and central to the sulfur-reducing metabolism of the organism.

**SirA – The enzyme.** The kinetic parameters and efficiency of the enzyme toward sulfite reduction have
not yet been determined. For this reason, the enzyme from M. tuberculosis was expressed in E. coli, purified to homogeneity (see Materials and Methods) and the kinetic constants were determined.

Obtaining pure SirA with the expected levels of siroheme cofactor (26) required co-expression of the E. coli CysG, a trifunctional protein that catalyzes the last three steps in siroheme biosynthesis (see Materials and Methods). The reduction reaction was monitored continuously, under anaerobic conditions, by following the change in absorbance at 684 nm associated with the oxidation of the electron donor, methyl viologen (39). Sulfide produced by SirA was removed rapidly by serine sulfhydrylase (from E. coli), which converts O-acetyl-\(l\)-serine and sulfide to cysteine (42). The removal of product ensures that product inhibition will not contribute significantly to the reaction, which simplifies extracting kinetic constants from the reaction progress curve (35). Under such conditions, slopes taken over sufficiently small regions (~ 3%) of the progress curve provide initial-rate measurements over an essentially continuously varying range of substrate concentration. The double-reciprocal plot of the SirA catalyzed reduction of sulfite is shown in Figure 5. The kinetic constants obtained by fitting the data are: \(K_m(\text{SO}_3^-) = 27 (\pm 1) \mu\text{M}\), \(k_{\text{cat}} = 0.17 (\pm .01) \text{ s}^{-1}\) (sulfite reduced to sulfide), or 1.0 \(\text{s}^{-1}\) (electrons consumed), and the catalytic efficiency, \(k_{\text{cat}}/K_m = 3.7 \times 10^4 \text{M}^{-1} \text{s}^{-1}\). It should be noted that, consistent with previous work (20), no enzymatic turnover was observed when sulfite was replaced by nitrite at concentrations as high as 3.0 mM.

**Growth phenotype of the Rv2393 deletion mutant.** Rv2393 is situated at the C-terminal end of sulfur reduction operon. This locale suggests that Rv2393, whose function is unknown, may be important for cysteine biosynthesis. To explore the ways in which Rv2393 might function in the cysteine biosynthetic pathway of M. smegmatis, an Rv2393 deletion strain was constructed (see Materials and Methods). The deletion removes the entire gene with the exception of short (140 and 53 nucleotide) stretches of sequence.
at the 5'- and 3'-edges of the coding region, respectively (see Fig. 3A). Growth of the deletion strain on sulfide or cysteine is indistinguishable from that of wild-type (Fig. 4B and C), suggesting that Rv2393 acts upstream of the enzymes that incorporate sulfide into cysteine (O-acetylserine sulfhydrylase (cysK1) and serine transacetylase (cysE)). In contrast, the doubling time of the mutant on sulfite or sulfate is ~ 2-fold slower than wild-type, Fig. 4B and C. Thus, the point-of-action of Rv2393 coincides with that of SirA – the reduction of sulfite.

The primary structure of Rv2393 clusters with an orthologous group of proteins from ancient phylogenies (COG2381.1) many of which are Type II cheletases - catalysts that insert divalent cations (Co$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, or Ni$^{2+}$) into modified tetrapyrroles to produce redox-sensitive cofactors including chlorophylls, vitamin B$_{12}$, heme, coenzyme F$_{430}$, and siroheme (43). Sulfite and nitrite reductases, which are often essential for the assimilation of sulfur and nitrogen, require siroheme to accomplish their redox chemistry. The linking of Rv2393 to the cheletase family and to sulfite reductase function provides a rational for the co-localization of sirA and Rv2393 and the common metabolic point-of-action of the proteins that they encode, which is that Rv2393 is the ferrocheletase that inserts Fe$^{2+}$ into sirohydrochlorin to produce siroheme cofactor that is necessary for SirA function.

To test the hypothesis that Rv2393 is, in fact, a ferrocheletase, plasmid-born Rv2393 was used to complement an E. coli ΔcysG mutant (302Δa, Table 1) in which ferrocheletase function is deleted. The genome of the mutant lacks cysG, which encodes siroheme synthase - a trifunctional protein that catalyzes the last three steps in siroheme biosynthesis: $i$, methylation of uroporphyrinogen III (at C-2 and C-7) to produce dihydrosirohydrochlorin (DHS); $ii$, oxidation of DHS to produce sirohydrochlorin (SHC); and $iii$, insertion of Fe$^{2+}$ into SHC to produce siroheme (28). The cysteine biosynthetic pathway is disrupted in
cysG-deleted strains at the point of insertion of iron into sirohydrochlorin. The cysteine auxotrophy that results from the inability to produce siroheme provides a nutrient-based selection for methyltransferase and/or cheletase function. It should be noted that the dehydrogenase function needed by these strains is provided by endogenous activity (44); thus, selecting for ferrochelatase function exclusively requires restoration of the methyltransferase activity in 302Δa, which is accomplished by transformation with pER247, a P15-origin plasmid that expresses the *Pseudomonas denitrificans* CobA, a uroporphyrinogen III methyltransferase that does not exhibit cheletase function (45, 46). The 302Δa strain harboring pER247 and pET23a with or without the *Rv2393* coding region were plated onto minimal-media plates containing either sulfate or cysteine as the sole source of sulfur (see *Ferrochelatase complementation* in *Material and Methods*) and incubated at 37 °C for 24 to 48 hours. The methyltransferase-competent strain (302Δa/pER247/pET23a) showed no detectible growth on sulfate as a sole source of sulfur. However, when transformed with a pET23A containing *Rv2393*, either from *M. tuberculosis* or *M. smegmatis*, the growth on the sulfate-containing plates was comparable to that of the cysG-complemented strain. The observed *Rv2393*-dependent transformation of the strain to cysteine prototrophy strongly supports that *Rv2393* encodes a ferrochelatase that is capable of inserting Fe$^{2+}$ into sirohydrochlorin to produce siroheme. Based on these findings, we have named *Rv2393*, *che1*, to indicate both its cheletase function and that it does not appear to be the only cheletase in the *M. tuberculosis* genome.

**Metabolic complementarity in sulfite and nitrite reduction.** Assimilatory nitrite and sulfite reductases, like those found in *M. smegmatis* and *M. tuberculosis*, share similar catalytic strategies, molecular architectures and substrate specificities. These enzymes are named on the basis of their relative catalytic efficiencies ($V_{\text{max}}/K_m$) for sulfite versus nitrite, and, interestingly, despite a greater overall efficiency toward nitrite, nitrite reductases often turnover faster with sulfite (i.e, $V_m(\text{SO}_3^-) > V_{\text{max}}(\text{NO}_3^-)$) (21). Thus,
the inability of the ΔsirA strain to grow on sulfite could reflect the inefficiency of the mycobacterial nitrite reductase toward sulfite, which has not been measured, and/or the level at which the enzyme is expressed in the organism when grown on ammonia (as is the case in the current studies).

Given that sulfite reductase (SirA) is remarkably inefficient toward nitrite (see above), it appears, from the M. smegmatis genome, that nitrite reductase (nirBD) is the organism’s sole means of obtaining ammonia from either nitrate or nitrite (47, 48). Ammonia is required for the biosynthesis of amino acids and, from there, nitrogen is drawn into the urea cycle, glutamate metabolism, pyrimidine biosynthesis and ultimately into every nitrogen containing compound in the cell (47). To assess the ability of nitrite reductase to reduce sulfite in-vivo, growth of the ΔsirA mutant on sulfate was assessed in a medium selected to optimize expression of nitrite reductase for the assimilation of nitrogen - M9 medium in which nitrate was substituted for ammonia at an equivalent concentration, 20 mM. Under this condition, all assimilated nitrogen must pass either through nitrite reductase or other, as yet unidentified, reductases in the cell. The substitution of nitrate for ammonia did not result in detectible growth of the organism on sulfate over a 70 hr period at 37 °C; however, replacement of sulfate by cysteine produced normal growth with nitrite (see Fig. 6A). Clearly, the nitrite reductase activity of the cell is sufficient to allow normal growth on metabolites that lie downstream of the point-of-action of SirA. Thus, nitrogen and sulfur metabolism are well isolated at the point of nitrite and sulfite reduction by what may ultimately prove to be pronounced differences in the substrate specificities of the enzymes that catalyze these reactions.

Unlike the ΔsirA mutation, which causes a no-growth phenotype, the Δche1 mutation results in slow growth of the organism on sulfate or sulfite. Thus, the orthogonality seen in the sulfite reduction step clearly does not extend to the siroheme cofactor, which can be provided to SirA from alternative
metabolic sources. Amelioration of slow growth by plasmid-born Che1 suggests that the endogenous siroheme pool(s) are in some way insufficient to achieve the levels of SirA activity needed for normal growth. Because nitrite reductase requires siroheme, it is plausible that growth of the organism on nitrite, as compared to ammonia, will up-regulate the cellular levels of siroheme; if so, the growth-rate of the \( \Delta \text{che1} \) mutant, when grown on sulfate or sulfite, will increase when the nitrogen source is switched from ammonia to nitrate. This is precisely what is observed (see Fig. 6B). Clearly, the perturbation of nitrogen metabolism caused by the ammonia-to-nitrate substitution has impacted sulfur metabolism at the intersection of sulfite reduction and ferrocheleatase function.
Discussion

The structure of SirA was determined recently, and the enzyme was shown to reduce sulfite (at a single sulfite concentration, 5.0 mM) under oxygen depleting conditions (excess sodium dithionite) (20). In the current work, the initial rate of sulfite reduction was studied under strict anaerobic conditions as a function of sulfite concentration, which yielded kinetic constants for the enzyme, \( k_{\text{cat}} = 0.17 \text{ s}^{-1} (\pm 0.1) \) (sulfite reduced) and \( K_m (\text{SO}_3^{-2}) = 27 \pm 1 \mu\text{M} \), that are in-line with previously published values for sulfite reductases from other organisms (21, 39). The value of \( k_{\text{cat}}/K_m \), \( 3.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \), indicates that SirA catalyzes \( \text{SO}_3^{-2} \) reduction with modest efficiency, and the inability to detect SirA-catalyzed reduction of nitrite at concentrations as high as 3.0 mM reveals that the efficiency of the enzyme toward \( \text{NO}_2^- \) is extremely low, and suggests that this reaction is not likely to be physiologically relevant.

High-density transposon-insertion mutagenesis in combination with micro-array mapping of the \( M.\ tuberculos\)is genome has indicated that \( \text{sirA} \) is important for optimal growth of the organism on minimal media containing sulfate as a sole source of sulfur (49). \( \text{SirA} \) was deleted in the present work and shown to be essential for the growth of \( M.\ smegmatis \) on sulfate or sulfite, regardless of whether nitrate or ammonia is used as a nitrogen source. These facts are of value not only for their contribution to the genetic and biochemical fundamentals of sulfur reduction in \( M.\ smegmatis \) and related organisms, but also for what they reveal about the orthogonality of the sulfur and nitrogen reducing pathways in these species. On this note, it may now be possible to more accurately annotate the sulfite vs nitrite substrate preferences of ferredoxin-dependent reductases based on primary sequence (20).

The common metabolic point-of-action of Rv2393 and SirA, the siroheme requirement for SirA
function, and the ability of Rv2393 to complement an *E. coli* ferrochelatase mutant, argue strongly that Rv2393, a protein with previously unidentified function, is a ferrochelatase, which we have named *che1*. The fact that the slow-growth phenotype of the Δ*che1* mutant is rescued by plasmid encoded Che1 or by growth on nitrate supports that the organisms growth rate limited by siroheme access. While nitrogen and sulfur metabolism are well isolated by what appear to be non-overlapping substrate specificities of the nitrite and sulfite reductases, these pathways do overlap at the level of siroheme production. It is interesting to note that a second CbiX-like open-reading frame (*rv0259c*) is found quite near the genes that encode nitrite reductase in *M. smegmatis*. Perhaps the siroheme pool(s) in the mutant grown on rate-limiting sulfur metabolites is simply too small to satisfy the metabolic demands placed on them; alternatively, access of SirA to the pool(s) could be limited by intrinsic specificities that bias delivery of the metallated-porphrin to particular recipients. It is exciting to consider that the sulfite-reduction growth-rate limited Δ*che1* strain presents the opportunity to establish linkage between sulfite reduction and metabolic sources of siroheme.
Acknowledgements

This work was supported by the National Institutes of Health Grant GM54469* and RO1 AI26170§.

We thank Professor Martin Warren and Evelyne Raux, Department of Biosciences, University of Kent, for kindly providing the *E. coli* cysG mutant strains, and Professor Ann-Francis Miller, Department of Chemistry and Biochemistry, University of Kentucky, for her generous guidance and support in executing the anaerobic, sulfite reductase assays.
Figure 1.

SO₄^{2-} → SO₄^{2-} → APS → ATP + ADP → sulfated metabolites

APS reductase (cysH) → ATP + GTP + P_i → PAPS

sulfuryl-transferases

sirA

Fe^{2+} → cheletase (Rv2393)

sulfite reductase (sirA)

acetylsulfenate

acetylsulfhydrylase (cysK1)

Fe^{2+} → cheletase (Rv2393)

feedback inhibition?

O-acetyl l-serine

AcCoA → l-serine

21
Figure 2.

A.

B.

TABLE 1. Primary sequence similarity of SirA from *M. smegmatis* and *M. tuberculosis*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Coding Region</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfite reductase</td>
<td>sirA</td>
<td>82</td>
<td>89</td>
</tr>
<tr>
<td>APS Reductase</td>
<td>cysH</td>
<td>70</td>
<td>78</td>
</tr>
<tr>
<td>Hypothetical</td>
<td>Rv2393</td>
<td>58</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 4.

A. 

\[ \text{OD}_{600} \]

\[ \Delta \text{sirA} \]

\[ \text{Sulfur Source} \]

- cysteine
- sulfide
- sulfite
- sulfate

B. 

\[ \Delta \text{rv2393} \]

C. 

\[ \text{Strain} \]

- Wild Type
- \( \Delta \text{nirA:comp sirA} \)
- \( \Delta \text{rv2393:comp rv2393} \)
Figure 5.

\[ \frac{1}{v} \text{ (mM s}^{-1}) = \frac{1}{[\text{SO}_3^{-}] (\mu\text{M})^{-1}} \]
Figure 6.

A.

B.
**TABLE 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-blue</td>
<td><em>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn 10Δ31 (TetR)]</em></td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21 (DE3) Rosetta pLysS</td>
<td>F´<em>ompT hsdSb (F' mB') gal dcm</em> (DE3) pLysSRARE2 (CamR)</td>
<td>Novagen</td>
</tr>
<tr>
<td>JR01</td>
<td>Rosetta pLysS carrying plasmid pJR1</td>
<td>This work</td>
</tr>
<tr>
<td>302Δa</td>
<td><em>E. coli cysG; Nir2 Lac</em> CysG°</td>
<td>(28)</td>
</tr>
<tr>
<td><em>E. coli cysG pER247</em></td>
<td>Strain 302Δa carrying the plasmid pER247 (pACYC184-lacq-Ptac-P. denitrificans cobA)</td>
<td>(28)</td>
</tr>
<tr>
<td>RP7</td>
<td><em>E. coli cysG pER247 containing plasmid pET23a M. tb Rv2393</em></td>
<td>This work</td>
</tr>
<tr>
<td>RP8</td>
<td><em>E. coli cysG pER247 containing plasmid pET23a M. smeg Rv2393</em></td>
<td>This work</td>
</tr>
<tr>
<td>HB101</td>
<td><em>E. coli K-12. FΔ(gpt-proA)62'leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xylI mtl-1 recA13</em></td>
<td>(29)</td>
</tr>
<tr>
<td><strong>M. smegmatis strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mc²155</td>
<td><em>ept-1</em></td>
<td>(30)</td>
</tr>
<tr>
<td>RPMS001</td>
<td>mc²155ΔsirA::hyg sacB</td>
<td>This work</td>
</tr>
<tr>
<td>RPMS002</td>
<td>mc²155ΔRv2393::hyg sacB</td>
<td>This work</td>
</tr>
<tr>
<td>RPMS004</td>
<td>RPMS001 carrying plasmid pRP15 (<em>M. smeg sirA complementation vector, Table 2</em>)</td>
<td>This work</td>
</tr>
<tr>
<td>RPMS005</td>
<td>RPMS002 carrying plasmid pRP16 (<em>M. smeg che1 complementation vector, Table 2</em>)</td>
<td>This work</td>
</tr>
<tr>
<td>Name</td>
<td>Description</td>
<td>Reference or source</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>p0004S</td>
<td>Cosmid containing the Hyg&lt;sup&gt;R&lt;/sup&gt;-SacB cassette</td>
<td>(34)</td>
</tr>
<tr>
<td>phAE159</td>
<td>Shuttle phasmid. TM&lt;sup&gt;4ts::pYUB328&lt;/sup&gt;</td>
<td>Kriakov and Jacobs</td>
</tr>
<tr>
<td>pMV261</td>
<td>&lt;sup&gt;kan&lt;/sup&gt;&lt;sup&gt;R&lt;/sup&gt;,&lt;sup&gt;colE1&lt;/sup&gt;,&lt;sup&gt;oriM&lt;/sup&gt;,&lt;sup&gt;aph&lt;/sup&gt;,&lt;sup&gt;Phsp60&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>pMV361</td>
<td>Intergrative vector&lt;sup&gt;hyg&lt;/sup&gt;&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(31)</td>
</tr>
<tr>
<td>pMS2391</td>
<td>Recombinant cosmid derived from p0004S containing the &lt;i&gt;M. smegmatis&lt;/i&gt; sir&lt;i&gt;A&lt;/i&gt; AES.</td>
<td>This study</td>
</tr>
<tr>
<td>pMS2393</td>
<td>Recombinant cosmid derived from p0004S containing the &lt;i&gt;M. smegmatis&lt;/i&gt; Rv2393 AES.</td>
<td>This study</td>
</tr>
<tr>
<td>pSKB4-9His-GST</td>
<td>Derived from pGEX-6P-1; Contains an N-terminal His-tag upstream to a GST tag.</td>
<td>(35)</td>
</tr>
<tr>
<td>pJR1</td>
<td>pSKB4-9His-GST carrying the &lt;i&gt;M. tuberculosis&lt;/i&gt; sir&lt;i&gt;A&lt;/i&gt; gene.</td>
<td>This study</td>
</tr>
<tr>
<td>pJR2</td>
<td>pJR1 with &lt;i&gt;E. coli&lt;/i&gt; &lt;i&gt;cysG&lt;/i&gt; cloned downstream of &lt;i&gt;sirA&lt;/i&gt;.</td>
<td>This study</td>
</tr>
<tr>
<td>pRP15</td>
<td>pMV261 carrying the &lt;i&gt;M. smeg&lt;/i&gt; sir&lt;i&gt;A&lt;/i&gt; gene.</td>
<td>This study</td>
</tr>
<tr>
<td>pRP16</td>
<td>pMV261 carrying the &lt;i&gt;M. smeg&lt;/i&gt; Rv2393 gene.</td>
<td>This study</td>
</tr>
<tr>
<td>pET23a</td>
<td>Cloning vector containing N-terminal His-tag.</td>
<td>Novagen</td>
</tr>
<tr>
<td>pRP17</td>
<td>pET23a carrying &lt;i&gt;M. tb&lt;/i&gt; Rv2393</td>
<td>This study</td>
</tr>
<tr>
<td>pRP18</td>
<td>pET23a carrying &lt;i&gt;M. smeg&lt;/i&gt; Rv2393</td>
<td>This study</td>
</tr>
<tr>
<td>pKK &lt;i&gt;E. coli&lt;/i&gt; &lt;i&gt;cysG&lt;/i&gt;</td>
<td>pKK223.3, an overexpression vector derived from pBR322 with &lt;i&gt;tac&lt;/i&gt; promoter, carrying &lt;i&gt;E. coli&lt;/i&gt; &lt;i&gt;cysG&lt;/i&gt;.</td>
<td>(28)</td>
</tr>
</tbody>
</table>
### TABLE 3. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Amplified Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBsirA&lt;sup&gt;a&lt;/sup&gt;F</td>
<td>ggaattcctatagtcgcaagggagaacccc</td>
<td><em>M. tuberculosis</em> sirA</td>
</tr>
<tr>
<td>TBsirA&lt;sup&gt;b&lt;/sup&gt;R</td>
<td>aagggaaagaagggcctagctgcaggtctctcgcggct</td>
<td></td>
</tr>
<tr>
<td>colicysG F</td>
<td>tttgcgcggacacgctgctgattacagaggggttttc</td>
<td><em>E. coli</em> cysG</td>
</tr>
<tr>
<td>colicysG R</td>
<td>ggcctagggtagtgcggccagaaccatcgttta</td>
<td></td>
</tr>
<tr>
<td>smgsirA F</td>
<td>tacagctgtgctgagacaggaacttcat</td>
<td><em>M. smegmatis</em> sirA</td>
</tr>
<tr>
<td>smgsirA R</td>
<td>cccagtctacatctcataacatctcagccgtg</td>
<td></td>
</tr>
<tr>
<td>smegRv2393 F</td>
<td>cgcagctggtgctgctgctgacaccc</td>
<td><em>M. smegmatis</em> Rv2393</td>
</tr>
<tr>
<td>smegRv2393 R</td>
<td>cccagtctacatctccataacatctcagccgt</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>ttttttttccataaattggtagatgcggagggcgccggtttgc</td>
<td>3' flanking region of <em>M. smegmatis</em> sirA</td>
</tr>
<tr>
<td>1'</td>
<td>ttttttttccataaattggtagatgcggagggcgccggtttgc</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ttttttttccataaattggtagatgcggagggcgccggtttgc</td>
<td>5' flanking region of <em>M. smegmatis</em> sirA</td>
</tr>
<tr>
<td>2'</td>
<td>ttttttttccataaattggtagatgcggagggcgccggtttgc</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ttttttttccataaattggtagatgcggagggcgccggtttgc</td>
<td>3' flanking region of <em>M. smegmatis</em> Rv2393</td>
</tr>
<tr>
<td>3'</td>
<td>ttttttttccataaattggtagatgcggagggcgccggtttgc</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>ttttttttccataaattggtagatgcggagggcgccggtttgc</td>
<td>5' flanking region of <em>M. smegmatis</em> Rv2393</td>
</tr>
<tr>
<td>4'</td>
<td>ttttttttccataaattggtagatgcggagggcgccggtttgc</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a,b</sup>F and R refer to forward and reverse primers.
**Figure Legends**

**FIG 1:** A segment of sulfur metabolism in mycobacteria. Red arrows and pneumonics identify the activities and genes associated with the sulfur reduction operon. Dotted arrows highlight steps that are the primary focus of this study. The cysteine feedback-inhibition loop is hypothesized based on similar inhibition in *S. typhimurium* (16) and *E. coli* (17), and complete conservation of the residues involved in the binding of cysteine (18, 19) across numerous organisms, including *M. tuberculosis* (19).

**FIG 2:** The sulfur-reduction operons of *M. smegmatis* and *M. tuberculosis*. (A) Schematic representation of the *M. smegmatis* and *M. tuberculosis sirA* operons. The double hash-mark in the *M. tb* diagram indicates a 5kb intergenic region between the operons. (B) ClustalW comparison metrics for homologous proteins encoded by the *M. smegmatis* and *M. tuberculosis* sulfur reduction operons (40).

**FIG 3.** Insertion of hygromycin resistance cassette into *sirA* and *Rv2393* in *M. smegmatis mc^2^155*. (A) The primer sets used for the construction of Southern blot probes and allelic exchange substrates. (B) Southern blot of genomic DNAs from wild-type (wt) and *ΔsirA* strains. *Ban* II digested genomic DNA was probed using a PCR fragment generated using primers 1 and 1′. The blot revealed that insertion of the resistance cassette caused the expected shift from 1.1 to 2.5 kb. (C) Southern blot of genomic DNAs from wild-type (wt) and *ΔRv2393* strains. *Msc* I digested genomic DNA was probed using a PCR fragment generated using primers 4 and 4′. Insertion of the resistance cassette resulted in the expected shift from 6.3 to 5.0 kb.
FIG 4: Growth of *M. smegmatis* mutants on various sulfur nutrients. (A) Growth of the Δ*sirA* mutant on minimal media containing cysteine (□), sulfide (●), sulfite (○) or sulfate (●) as the sole sulfur source. Generation times for growth on cysteine and sulfide are 7.5 and 6.7 hours, respectively; no growth was observed in media containing sulfite or sulfate. (B) Growth of the Δ*Rv2393* mutant on the same sulfur sources as in A. The generation time for the mutant was 10 hours when the media contained cysteine or sulfide. Cells fed sulfate or sulfite both exhibited a steady-state growth with a generation time of 19 hours. (C) To demonstrate that the growth effects of the deletions are due solely to lack of expression of the intended coding regions, the growth on sulfate of the deletion strains complemented with plasmids that express the wild-type gene was compared to that of the wild-type strain containing empty vector. The doubling times of the three strains were extremely similar: wild type (○), 8.8 hr; Δ*sirA*: comp *sirA* (□), 8.6 hrs; Δ*Rv2393A*: comp *Rv2393* (●), 8.5 hrs. Growth protocols are described in *Materials and Methods*. Each data point represents the average of three experiments - single standard deviation units (not shown) are comparable to the diameter of the symbols representing the averaged values.

**Figure 5: Initial-rate determination of the Michaelis constants for the SirA-catalyzed reduction of sulfite.** Sulfite reduction was monitored continuously (under stringent, anaerobic conditions) via the change in absorbance at 684 nm associated with the oxidation of the electron donor, methyl viologen (see *Materials and Methods*). The assay conditions were as follows: sulfite (13 μM), reduced methyl viologen (250 μM), O-acetyl-l-serine (30 μM), SirA (1.3 μM), O-acetyl-l-serine sulfhydrolase (1.2 μM), 50mm Hepes/K+ pH 8.0, T = 25 (±3) °C. The reaction rate is given in terms of sulfite reduced per unit time, μM/s⁻¹. The reduction of one equivalent of sulfite requires the oxidation of six equivalents of methyl viologen.
FIG 6: Growth of *M. smegmatis* mutants on nitrate. (A) Growth of the Δ*sirA* mutant on minimal media containing nitrate as the sole nitrogen source and either cysteine (○) or sulfate (●) as the sole sulfur source. Generation times for growth on cysteine was 7.1 hours, and as no growth was observed in media containing sulfate. (B) Growth of the Δ*che1* mutant on the same nitrogen and sulfur sources as in A. Generation times for growth on cysteine or sulfate were virtually identical, 7.3 hours. Growth protocols are described in *Materials and Methods*. Each data point represents the average of three experiments - single standard deviation units (not shown) are comparable to the diameter of the symbols representing the averaged values.
References


