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The *Mycobacterium tuberculosis* CysQ phosphatase modulates the biosynthesis of sulfated glycolipids and bacterial growth

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Abstract

CysQ is a 3'-phosphoadenosine-5'-phosphatase that dephosphorylates intermediates from the sulfate assimilation pathway of *Mycobacterium tuberculosis* (*Mtb*). Here, we demonstrate that *cysQ* disruption attenuates *Mtb* growth *in vitro* and decreases the biosynthesis of sulfated glycolipids but not major thiols, suggesting that the encoded enzyme specifically regulates mycobacterial sulfation.

Keywords

Sulfur metabolism; CysQ; Phosphatase; Sulfation; Glycolipids

Sulfur-containing metabolites from *Mycobacterium tuberculosis* (*Mtb*) are integral to the virulence of this global pathogen.^{1,2} Reduced sulfur compounds, such as cysteine and mycothiol, the major thiol-containing small molecule of mycobacteria, confer resistance to oxidative stress.^{3–6} Collectively, they are believed to facilitate *Mtb* survival during the chronic phase of infection in mice.³ Sulfated compounds are also intimately linked to mycobacterial virulence.^{7,8} The sulfated glycolipid Sulfolipid-1 (SL-1) is exclusively synthesized by pathogenic mycobacteria,⁹ while its biosynthetic precursor SL₁₂₇₈ has been shown to elicit a potent immune response from human tuberculosis patients.¹⁰ Biosynthesis of these important molecules relies on the sulfate assimilation pathway.¹¹

The sulfate assimilation pathway of *Mtb* (Scheme 1) begins with the conversion of sulfate to adenosine 5'-phosphosulfate (APS) in a reaction catalyzed by ATP sulfurylase.¹² APS is subsequently metabolized via one of two pathway branches: it is either reduced to sulfite by

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APS reductase for the biosynthesis of essential reduced sulfur metabolites (reductive branch), or phosphorylated by APS kinase to give 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the universal sulfate donor in the cell (sulfation branch).¹¹ PAPS is a substrate for sulfotransferase enzymes that transfer its 5'-sulfate group onto organic metabolites.⁹ A byproduct of this reaction is 3'-phosphoadenosine 5'-phosphate (PAP), a competitive inhibitor of sulfotransferase activity.¹³ We previously demonstrated that *Mtb* encodes a 3'-phosphoadenosine-5'-phosphatase termed CysQ that is capable of dephosphorylating PAP and its sulfated counterpart.¹⁴ Notably, homologous enzymes have been shown to significantly affect the biosynthesis of sulfur-containing metabolites in *Escherichia coli* and *Saccharomyces cerevisiae*.^{15–17} We hypothesized that CysQ might similarly regulate sulfur metabolism in *Mtb* by modulating the levels of these key pathway intermediates.

To explore the role of this enzyme in *Mtb* sulfur metabolism, we generated a *cysQ*-deficient mutant, $\Delta cysQ$, from *Mtb* strain Erdman by homologous recombination as previously described.¹⁸ The mutant was designed such that 340 internal base pairs of the native *cysQ* gene (amino acids 61 through 173) were replaced with a hygromycin resistance cassette. The $\Delta cysQ::cysQ$ complementation strain was created by cloning the *cysQ* gene from *Mtb* strain Erdman with an additional 350 base pairs at its 5' end and 80 base pairs at its 3' end into the mycobacterial expression vector pMV306, a derivative of the pMV361 vector¹⁹ with a multiple cloning site in place of the expression cassette. The resulting plasmid was electroporated into $\Delta cysQ$ and transformants were selected on Middlebrook 7H10 agar plates containing 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco), 0.5% glycerol, 0.05% Tween-80, and 25 mg/mL kanamycin.

We evaluated the effect of *cysQ* disruption on the growth kinetics of *Mtb* by monitoring the growth of synchronized wild-type, $\Delta cysQ$, and $\Delta cysQ::cysQ$ *Mtb* for a period of 5 d. Cultures were prepared using Middlebrook 7H9 medium supplemented with 10% OADC, 0.5% glycerol, and 0.05% Tween-80 and incubated in vented shaker flasks at 37 °C on an orbital shaker. Bacterial growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) with a Thermo Scientific Genesys 20 Visible Spectrophotometer. The mutant strain exhibited attenuated growth relative to wild-type, and this phenotype was reverted by complementation (Figure 1). Because the growth medium used in these studies is devoid of reduced sulfur species, survival of the cultured bacteria relies on the conversion of sulfate to essential reduced sulfur compounds via the sulfate assimilation pathway. We therefore considered the possibility that the observed attenuation of the mutant strain might reflect an impaired biosynthesis of reduced sulfur compounds in the absence of CysQ. To test this hypothesis, we grew $\Delta cysQ$ in the presence of either 2 mM cysteine or methionine. Notably, these sulfur-containing amino acids have previously been shown to restore wild-type growth to the APS reductase mutant of *Mtb*, which is deficient in the *de novo* synthesis of reduced sulfur species and consequently unable to grow in 7H9 medium.³ In contrast, neither cysteine nor methionine was able to rescue the growth of $\Delta cysQ$ (Figure 1), indicating that a deficiency in reduced sulfur compounds is not responsible for the growth attenuation of the mutant strain.

To provide quantitative support for this hypothesis, we sought to compare the concentrations of major thiol-containing compounds produced by wild-type, $\Delta cysQ$, and $\Delta cysQ::cysQ$ *Mtb* using the monobromobimane method. Extracts from exponentially growing bacteria were analyzed for mycothiol, coenzyme A (CoA), cysteine, and hydrogen sulfide, a derivative of iron-sulfur proteins, as previously described.²⁰ Briefly, triplicate cultures of each strain were grown to log-phase in 7H9 medium, and the OD₆₀₀ of each was determined. The cultures were divided in two and pelleted for 5 min at 3500 rpm. Samples for thiol analysis were resuspended in 50% (v/v) acetonitrile in 20 mM HEPES (pH 8.0) containing 2 mM monobromobimane (Invitrogen). The remaining samples were resuspended in 50% (v/v)

acetonitrile in 20 mM HEPES (pH 8.0) containing 5 mM *N*-ethylmaleimide (Sigma) prior to treatment with monobromobimane to serve as a thiol-blocked control. Bacteria were incubated for 15 min at 60 °C, and then pelleted for 5 min at 14000 rpm. The supernatants were twice sterilized by filtration prior to removal from the biosafety level 3 facility and subsequently stored at –80 °C until analysis. High-performance liquid chromatography was used to determine the mycothiol, hydrogen sulfide, CoA and cysteine content of each sample.^{5,21} Mycothiol, the most abundant thiol in mycobacteria,²² was present at similar levels in all three strains (Figure 2A). Further, hydrogen sulfide and CoA content were not affected by *cysQ* disruption (Figures 2B and 2C). Cysteine levels were very low in all samples (i.e., less than or equal to 0.1 nmol per 10⁹ cells) as found previously for wild-type *Mtb*.²⁰ Mycothiol, hydrogen sulfide, and CoA concentrations were also similar to those previously reported in the literature.²² Only the CoA content of $\Delta cysQ::cysQ$ deviated significantly from the other two strains in this analysis. This deviation is most likely a consequence of the variation in cell density among the harvested strains, which has previously been shown to affect CoA levels.²⁰ However, the overall homogeneity in the thiol composition of wild-type, $\Delta cysQ$, and $\Delta cysQ::cysQ$ *Mtb* provides further evidence that CysQ does not regulate the biosynthesis of reduced sulfur species in these bacteria.

To determine if the enzyme might instead influence the biosynthesis of sulfated metabolites, we evaluated the relative levels of two prominent sulfated glycolipids in wild-type, $\Delta cysQ$, and $\Delta cysQ::cysQ$ *Mtb* by metabolic labeling and autoradiography (Figure 3). Synchronized cultures were grown to log-phase, normalized by cell density, pelleted for 5 min at 3500 rpm and resuspended in 10 mL of PBS containing 1 mg/mL sodium acetate. Each sample was labeled with 100 μ Ci of sodium [³⁵S]sulfate (PerkinElmer) for 16–18 h at 37 °C in a rotary shaker. Bacteria were pelleted for 5 min at 3500 rpm and extracted with chloroform:methanol (1:1). Equal volumes of total lipid extracts from each strain were analyzed by thin-layer chromatography (TLC) (60:12:1 chloroform:methanol:water) for the presence of SL-1 and its biosynthetic precursor SL₁₂₇₈. Phosphorimaging followed by densitometry analysis using ImageJ software was used to quantify radioactivity. Spots corresponding to SL-1 and SL₁₂₇₈ were assigned based on previously published TLC assays²³ and confirmed using radiolabeled extracts from the SL-1 biosynthetic mutants $\Delta stf0$ and $\Delta mmpL8$.^{24,25} Importantly, the $\Delta stf0$ strain lacks all known precursors in the SL-1 biosynthetic pathway, including SL₁₂₇₈, whereas the $\Delta mmpL8$ strain accumulates SL₁₂₇₈ in the cell wall.^{25–27} The integrated density of each spot was normalized to the background density of the TLC plate and reported as a percentage of the total integrated density of its respective lane. The averaged data from three independent experiments revealed that both SL-1 and SL₁₂₇₈ are present at reduced levels in $\Delta cysQ$ (Figure 3C); the percentage of these metabolites in the mutant strain was approximately one-third that of wild-type *Mtb*. Complementation restored SL-1 to wild-type levels and increased production of SL₁₂₇₈, confirming that the reduction of these metabolites in the mutant can be attributed to the loss of a functional CysQ.

The ability of CysQ to modulate mycobacterial sulfation most likely lies in its ability to degrade PAP, a byproduct and competitive inhibitor of sulfotransferase activity.^{13,14} Inhibition of a CysQ homologue from *S. cerevisiae* has been shown to significantly increase the intracellular concentration of PAP.¹⁷ Thus, in the absence of a functional CysQ, sulfotransferase activity may be subject to inhibition by PAP accumulation. Inhibition of Stf0, the sulfotransferase that catalyzes the first committed step of SL-1 biosynthesis²⁶, would reduce levels of SL-1 and its precursor SL₁₂₇₈, which is consistent with our findings. However, our attempts to determine the effect of *cysQ* disruption on the concentration of PAP in *Mtb* lysates were precluded by the complexity of the cellular extracts and low abundance of this metabolite.

Importantly, with the exception of SL-1 and SL₁₂₇₈, the overall labeling of sulfur-containing metabolites in wild-type and Δ cysQ extracts by [³⁵S]sulfate appears similar by TLC (Figure 3B), suggesting that *cysQ* disruption only affects a small subset of sulfur-containing compounds. Further, a recent study by Movahedzadeh *et al.*²⁸ established that the overall lipid content of *Mtb* is not affected by the disruption of *cysQ*, indicating that the observed changes in the content of sulfated glycolipids in the Δ cysQ strain do not reflect a global reduction in glycolipid production, but rather a specific decline in their biosynthesis.

While there is a clear biochemical basis for the correlation between CysQ activity and mycobacterial sulfation, the relationship between mycobacterial sulfation and growth is not as easily explained. One possibility is that the disruption of *cysQ* inhibits the biosynthesis of one or more sulfated biomolecules required for wild-type growth. Interestingly, the SL-1 biosynthetic mutants Δ stf0 and Δ mmpL8 exhibit wild-type growth in liquid media, indicating that SL-1 is dispensable for *Mtb* growth *in vitro* (S. Gilmore and C. Bertozzi, unpublished data).²⁵ These findings suggest that the loss of another sulfated metabolite may be responsible for the growth attenuation of Δ cysQ. Alternatively, a second possibility is that the growth attenuation of Δ cysQ does not arise from the loss of a sulfated biomolecule, but from the accumulation of the sulfate donor PAPS. In the absence of CysQ, PAP inhibition of sulfotransferase activity could lead to a build-up of this substrate, which previous studies suggest is cytotoxic to *E. coli*.¹⁵ A third possibility is that the pleiotropic effects of PAP accumulation may be accountable for the growth defect of Δ cysQ. In addition to inhibiting sulfotransferases, PAP is known to negatively regulate oligoribonucleases,²⁹ which degrade small ribonucleotides, and 4'-phosphopantetheinyl transferases,³⁰ which activate acyl carrier proteins during the biosynthesis of fatty acids and secondary metabolites. Thus, the disruption of *cysQ* may have far-reaching consequences that extend beyond the confines of sulfur metabolism into other essential bacterial processes.

Though questions remain regarding the effects of CysQ on bacterial growth, its influence over mycobacterial sulfur metabolism is now more clearly defined. Loss of the enzyme decreases the biosynthesis of key sulfated metabolites, while the thiol composition of *Mtb* remains largely unchanged. These findings, in accordance with the established biochemical activity of the enzyme, are consistent with the inhibition of sulfotransferase activity and suggest CysQ may specifically regulate the sulfation branch of the sulfate assimilation pathway, a nexus of biosynthetic enzymes whose metabolic potential is still largely unknown.

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Figure 1.

Disruption of *cysQ* attenuates *Mtb* growth in vitro. Wild-type ($\blacklozenge$), $\Delta cysQ$ ($\blacksquare$), and $\Delta cysQ::cysQ$ ($\blacktriangle$) *Mtb* were grown in Middlebrook 7H9 medium at 37 °C on an orbital shaker. Bacterial growth was monitored over a period of 5 d by measuring optical density (OD_{600}). The addition of either 2 mM cysteine (long dash) or methionine (short dash) to the growth medium did not restore $\Delta cysQ$ growth. These results represent the averaged data from triplicate cultures.

Figure 2.

CysQ does not affect the biosynthesis of major thiols. The A) mycothiol, B) hydrogen sulfide, and C) coenzyme A content of wild-type, $\Delta cysQ$, and $\Delta cysQ::cysQ$ *Mtb* was determined by extracting cells with 50% (v/v) acetonitrile in 20 mM HEPES (pH 8.0) containing 2 mM monobromobimane, followed by HPLC analysis of cellular extracts. These results represent the averaged data from extracts of triplicate cultures. *, $P < 0.01$ by two-tailed Student's *t* test.

Figure 3.

Loss of CysQ decreases the biosynthesis of the sulfated glycolipids Sulfolipid-1 and SL₁₂₇₈. A) Chemical structures of Sulfolipid-1 and SL₁₂₇₈. B) Total lipid extracts from wild-type, $\Delta cysQ$, $\Delta cysQ::cysQ$, $\Delta stf0$, and $\Delta mmpL8$ *Mtb* labeled with [³⁵S]sulfate were analyzed by TLC and phosphorimaging. C) Relative levels of Sulfolipid-1 and SL₁₂₇₈ were determined by densitometry. The integrated density of each spot was normalized to the background density of the TLC plate and reported as a percentage of the total integrated density of its respective lane. These results represent the averaged data from three independent experiments. *, $P < 0.05$ by two-tailed Student's *t* test.

Scheme 1.

The sulfate assimilation pathway of *Mtb*. Inorganic sulfate from the host is converted to adenosine 5'-phosphosulfate (APS) by ATP sulfurylase. APS is either reduced to sulfite by APS reductase for the biosynthesis of essential reduced sulfur metabolites (reductive branch), or phosphorylated by APS kinase to generate 3'-phosphoadenosine 5'-phosphosulfate (PAPS), a substrate for sulfotransferases (sulfation branch). 3'-Phosphoadenosine 5'-phosphate (PAP), a byproduct of sulfotransferase reactions, is degraded by the PAP(S) phosphatase CysQ, which also converts PAPS to APS.