Tuberculosis 115 (2019) 63–66

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: www.elsevier.com/locate/tube

Mycobacterium tuberculosis cysteine biosynthesis genes *mec+-cysO-cysM* confer resistance to clofazimine

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ARTICLE INFO

Keywords:
Cysteine biosynthesis
Mycobacterium tuberculosis
Clofazimine resistance

ABSTRACT

The *Mycobacterium tuberculosis mec+-cysO-cysM* gene cluster was shown to be part of a novel cysteine biosynthesis pathway in vitro, but little is known about its essentiality or role in *M. tuberculosis* physiology. In this study, we generate a knock out of the *mec+-cysO-cysM* gene cluster in *M. tuberculosis* and show that the gene cluster is not essential under a variety of conditions, suggesting redundancy in pathways for cysteine biosynthesis in *M. tuberculosis*. The cysteine biosynthesis gene cluster is essential for resistance for clofazimine, a peroxide-producing anti-leprosy drug. Therefore, although under most conditions the pathway is not essential, it likely has an important role in defense against oxidative stress in *M. tuberculosis*.

1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), caused ten million infections and 1.6 million deaths in 2017 (WHO, 2018). It is estimated that nearly one-third of the world's population is infected with TB, many of whom are latently infected and show no signs of active disease. *M. tuberculosis* remains latent in many individuals because the human immune system cannot completely clear the bacterium, sometimes even with the aid of antibiotics. When the immune system is compromised, the persistent bacteria can reactivate and transmission becomes a risk.

Macrophages, the major cellular host for *M. tuberculosis*, use oxidative stress in the form of reactive oxygen/nitrogen intermediates (ROI/RNI) to alter *M. tuberculosis*'s redox balance, to damage macromolecules and to defeat the bacterium [1,2]. *M. tuberculosis* is able to counteract these host-derived ROI/RNI by protecting oxygen-sensitive proteins and molecules using thioredoxins and mycothiol [1,3], for example, and can survive in the host for years. The activity of these oxidative stress-defending molecules is dependent on the thiol group of the amino acid cysteine: the active site of thioredoxins contains a CXXC motif [4], and the critical component of mycothiol is also the thiol group of cysteine [5,6], as two examples. To perform normal cellular functions and to defend against the oxidative stress imposed by the host cell, *M. tuberculosis* must make the amino acid cysteine both under normal growth conditions and under conditions of stress.

The biosynthesis of cysteine in *M. tuberculosis* has not been studied extensively. Analysis of the *M. tuberculosis* genome suggests that *M. tuberculosis* could biosynthesize cysteine using several routes. The genome contains a classical cysteine synthase *cysK* gene (*rv2334*) directly upstream of a putative serine acetyltransferase (*cysE, rv2335*), suggesting a role for direct sulfuration for cysteine biosynthesis in *M. tuberculosis* (Fig. 1A). In addition, another putative cysteine synthase gene, *cysK2* (*rv0848*), has been shown to have S-sulfocysteine synthase activity in vitro [7]; however, its function in *M. tuberculosis* remains unclear. *Rv3684* is also proposed to encode a cysteine synthase, but no in vitro work has confirmed this annotation and the genomic neighbors do not support a role of this gene in cysteine biosynthesis. Finally, *cysM* (*rv1336*), an O-phosphoserine sulfhydrylase [8,9], is clustered in an operon (*rv1334-rv1336*, *mec+-cysO-cysM* or *mcc* from hereon) with genes shown in vitro to be involved in the biosynthesis of cysteine using a novel alternative pathway [10] (Fig. 1B). Instead of using the oxidation-prone sulfide as the direct sulfur donor for cysteine biosynthesis, this pathway uses a protein-bound thiocarboxylate as the donor in vitro. This novel pathway was the only putative cysteine biosynthetic pathway upregulated by *sigH* in response to oxidative stress [11]. In addition, a five residue C-terminal peptide was shown to protect the CysM aminoacrylate reaction intermediate from hydrogen peroxide reactivity in vitro [12]. It is proposed that this pathway could provide *M. tuberculosis* with a way to make cysteine that is stable to oxidative insults [10].

The *M. tuberculosis* genes *mec+*, *cysO* and *cysM* are predicted not to be essential under standard growth conditions [13]; however, using a TraSH screen, the strain with a transposon mutant in *mec +* is believed to have a survival defect in primary murine macrophages [14].

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https://doi.org/10.1016/j.tube.2019.02.002
Received 22 October 2018; Received in revised form 25 January 2019; Accepted 3 February 2019

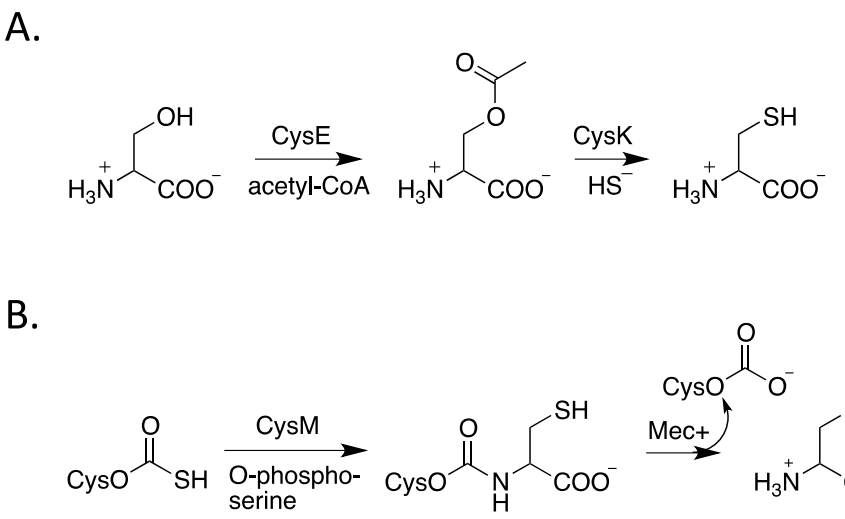

Fig. 1. Biosynthetic pathway encoded by *cysE-cysK* (A) and *mec+-cysO-cysM* (*mcc* cluster) (B) in Mtb. (A). CysE catalyzes the transfer of an acetyl group on serine, and CysK catalyzes the addition of sulfide to form cysteine. (B). The protein-bound thiocarboxylate on CysO is the sulfur donor for CysM-mediated addition of cysteine onto CysO. Mec + hydrolyzes the C-terminal residue, releasing cysteine.

Interestingly, a homolog of *mec* + restored the nutritional requirement for methionine and cysteine in a mutant strain of *Streptomyces kasugaensis* [15]. This finding suggests that for at least one actinomycete, this cluster could be responsible for all of the cellular cysteine and is essential. More recently, inhibitors of CysM were identified and were shown to impart ~2–3 $\log_{10}$ survival defect on nutrient-deprived *M. tuberculosis* [16], suggesting that this pathway is important under starvation conditions. However, transcriptional profiling by Betts et al. [17] suggests that these genes are down-regulated under nutrient starvation.

To determine the role of the *mcc* cluster in *M. tuberculosis*, we used homologous recombination to generate a knockout of the cluster and determined its essentiality in vitro under conditions of stress, as well as its susceptibility to a variety of antibiotics. Our results confirm that the *mcc* cluster is not essential under standard in vitro growth conditions and in a variety of stresses we imposed, including nutrient starvation; however, the *mcc* cluster confers resistance to the ROS-generating antileprosy drug clofazimine, suggesting a role in cellular redox homeostasis.

2. Results

2.1. mcc is not essential under standard culture conditions in vitro

To determine the essentiality of *mcc*, we replaced most of the *mcc* cluster with a hygromycin resistance cassette using homologous recombination. We obtained many hygromycin-resistant colonies, suggesting that the cluster is not essential under standard culture conditions in vitro. Both PCR and Southern blot confirmed the replacement (Fig. S1a and b). We generated a complementation vector in the integrative vector pMV306, which contains an *hsp60* promoter. When compared to WT, the *mcc* cluster deletion and the complemented strains did not show any defects in growth in standard 7H9 medium (Fig. 2), which does not contain cysteine.

2.2. The mcc cluster is not essential upon nutrient-deprivation

Recently, CysM was the target of a screening campaign that identified a few selective inhibitors [16]. These inhibitors had μM minimal inhibitory concentration (MIC) against replicating *M. tuberculosis* and resulted in ~2–3 $\log_{10}$ reduction in CFU in a PBS model of nutrient deprivation in *M. tuberculosis* [16]. This activity was rescued by the addition of cysteine. To determine whether the *mcc* cluster is important under conditions of nutrient deprivation, we tested the survival of the WT parental strain, *Δmcc* and its complement after six weeks of starvation in PBS (Fig. S2). We observed no defect in the survival of the *mcc* deletion mutant under these conditions.

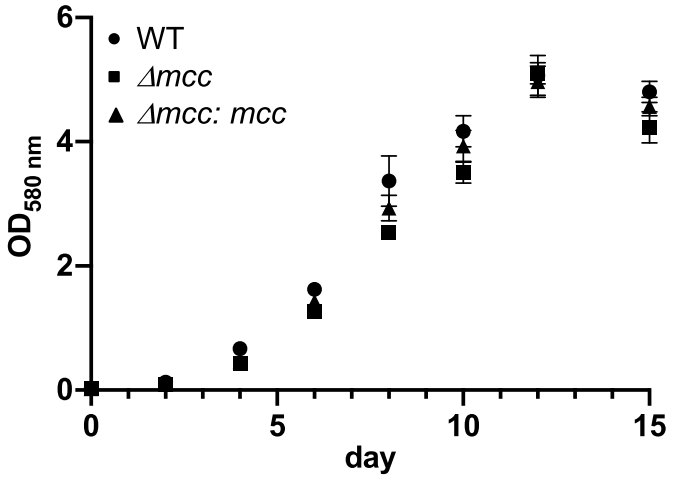

Fig. 2. Growth curve of WT, Δ*mcc* and complemented strains. Strains were grown in 7H9-tyl as stationary cultures. Data are the average of three independent cultures for each strain and are representative of three independent experiments.

2.3. MIC analysis of the mcc cluster deletion

Our hypothesis is that the *mcc* cluster is important for *M. tuberculosis* under conditions of stress, in particular oxidative stress. We determined the MIC values of a variety of antibiotics to the *mcc*-deleted strain, shown in Table 1. The WT, *Δmcc* and complement strains had similar MIC for all of the compounds tested except for clofazimine. When exposed to clofazimine, cells lacking the *mcc* cluster had about half the

Table 1
Activity (MIC_{90}) of compounds against strains (μM).

	WT	*Δmcc*	*Δmcc: mcc*
Rifampicin	0.078 ± 0.02	0.13 ± 0.04	0.11 ± 0.05
Isoniazid	0.12 ± 0.02	0.18 ± 0.06	0.12 ± 0.02
Moxifloxacin	0.26 ± 0.1	0.39 ± 0	0.26 ± 0.1
Streptomycin	1.1 ± 0.5	1.7 ± 0	0.86 ± 0
Diamide	15000 ± 0	15000 ± 0	15000 ± 0
Menadione	160 ± 0	130 ± 40	210 ± 90
Clofazimine	1.3 ± 0	0.66 ± 0	4.4 ± 1.5

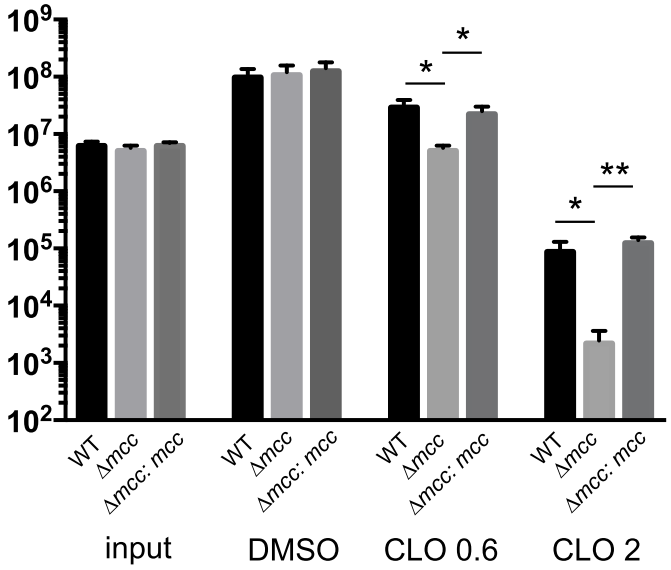

Fig. 3. Susceptibility of the Mtb Δ*mcc* strain to clofazimine. The strains were incubated for 13 days with the indicated amount of clofazimine (in μM). Data are representative of three independent experiments. (**) P value < 0.001, (*) P value < 0.05 using two-tailed unpaired *t*-test analysis.

MIC compared to WT; however, the complement had a reproducible seven-fold increase in MIC compared to *Δmcc*.

2.4. *M. tuberculosis* lacking the mcc cluster are susceptible to clofazimine

To investigate the susceptibility of *M. tuberculosis* lacking the *mcc* cluster to clofazimine in more detail, we incubated the WT, *Δmcc* and complement with 0.6 μM (0.3 μg/mL) or 2 μM (1 μg/mL) clofazimine, and then plated for CFU following 13 days of exposure. Incubation with 0.6 μM clofazimine for 13 days of exposure led to about 0.5 $\log_{10}$ survival defect of the *Δmcc* strain compared to WT (Fig. 3). Upon exposure to 2 μM clofazimine, all strains showed a survival defect compared to the input; however, the *Δmcc* strain had ~1–1.5 log10 survival defect compared to WT. This defect was complemented upon addition of the *mcc* cluster on a plasmid.

3. Discussion

Exposure to ROS is a consequence of the intracellular lifestyle of *M. tuberculosis*. *M. tuberculosis*'s defense against ROS is critical to its survival under these conditions. Because much of *M. tuberculosis*'s defense against ROS involves the amino acid cysteine, understanding its biosynthesis in *M. tuberculosis* is necessary to understanding potential vulnerabilities to these insults.

We have shown that the cysteine biosynthesis *mcc* cluster is not essential under standard growth conditions in vitro. The non-essentiality of the cluster suggests that *M. tuberculosis* has more than one way to make cysteine, as evidenced by the fact that the *M. tuberculosis* genome encodes several putative cysteine biosynthetic genes. The *mcc* cluster is also not essential under conditions of nutrient starvation, which is supported by transcriptional profiling studies [17] showing that these genes are down-regulated under nutrient starvation. It is possible that the CysM inhibitors that were recently identified to be cidal against starved *M. tuberculosis* [16] have more targets under these conditions. It is also possible that there are compensatory changes in our *mcc* knockout that enable the strain to adapt to the lack of cysteine, which may not be observed in inhibition experiments.

Clofazimine is a riminophenazine anti-leprosy drug used in combination with rifampicin and dapsone. After a successful study from Bangladesh [18] where addition of clofazimine to a regime cured drug-resistant TB (DR-TB) in many patients who were treated, and other studies showing efficacy against DR-TB [19,20], clofazimine has been repurposed for treatment of TB. In mycobacteria, it is thought to be a prodrug that competes with menaquinone, a key electron acceptor, for reduction by type 2 NADH-quinone oxidoreductase (NDH-2). Spontaneous reoxidation by molecular oxygen generates ROS, which results in cell death [21,22]. The Δ*mcc* strain showed increased sensitivity to clofazimine compared to WT and complement strains, supporting the hypothesis that the cluster plays a role in defense against oxidative stress in *M. tuberculosis*. *M. tuberculosis* lacking the *mcc* cluster is not more sensitive than WT or the complement strain to hydrogen peroxide (Fig. S3) or *tert*-butyl peroxide (not shown). It is unclear why other compounds that are believed to generate ROS had no effect on the *mcc* cluster deletion; perhaps the type of ROS generated and/or the duration of the ROS generated plays a role. In addition, the presence of multiple oxidative stress defense mechanisms in *M. tuberculosis* likely affects sensitivity of this strain to specific inducers of ROS. Finally, is possible that the sensitivity of the Δ*mcc* strain to clofazimine is independent of its role in countering ROS.

M. tuberculosis, an organism that is likely constantly under oxidative stress, has multiple mechanisms to defend against this stress. Since the biosynthesis of cysteine is critical for defense against oxidative stress, it makes sense that *M. tuberculosis* has multiple and somewhat redundant pathways to make cysteine. In fact, the redundancy of pathways highlights the critical role cysteine plays in *M. tuberculosis* physiology. Our data reveals that there is likely more than one route to make cysteine in *M. tuberculosis*, and that the *mcc* cluster plays a role in the defense against specific oxidative stress in *M. tuberculosis*. These findings open the door for investigating the redundancy of cysteine biosynthetic pathways in *M. tuberculosis* and for increasing the efficacy of clofazimine in treating DR-TB by targeting *M. tuberculosis*'s defense against oxidative stress.

4. Materials and methods

4.1. Growth conditions

E. coli was grown in LB broth (Difco) or LB agar (Difco); *M. tuberculosis* H37Rv was grown in Difco Middlebrook 7H9 (BD Biosciences) supplemented with 0.2% glycerol, 10% ADN (0.5% albumin (Roche), 0.2% dextrose, 0.085% sodium chloride) and 0.02% tyloxapol (7H9-tyl) or Difco Middlebrook 7H11 or 7H10 (BD Biosciences) supplemented with 0.2% glycerol and 10% v/v OADC (BD Biosciences) (7H11). The following antibiotics were used: hygromycin B (50 μg/mL (Mtb) and 150 μg/mL (*E. coli*)); kanamycin (25 μg/mL (Mtb) and 100 μg/mL (*E. coli*)).

4.2. Knockout strain and complement construction

The *mcc* cluster from *rv1334-rv1336* (*mec* + *-cysM*) was replaced in H37Rv with a hygromycin-resistance cassette by allelic exchange using recombineering. ~800 bp of genomic region upstream and downstream of the *mcc* cluster was amplified using primer pairs 6 and 8 (upstream) and 5 and 9 (downstream) (Table S1) and cloned to flank a gene encoding hygromycin resistance. PCR (Table S1 primers 3, 4) was used to generate the hygromycin resistance gene flanked by ~500 bp of upstream and downstream *mcc* cluster. The ~3.2 kb PCR product was gel purified and electroporated into competent nitrile-induced H37Rv with the pNIT(kan):RecET-SacB plasmid [23] to allow for recombineering. Transformants were plated on 7H11 agar supplemented with hygromycin B. Candidate clones were tested for sensitivity to kanamycin and two were tested for *mcc* cluster deletion by PCR and Southern blot. For Southern blot, DNA from WT and *Δmcc* cluster was digested with MluI (New England Biolabs) and fragments were separated on an agarose gel. The DNA was transferred to a nylon membrane and the membrane was probed with a 400 bp labeled fragment

generated with primers 12 and 13 (Table S1) using ECL direct nucleic acid labeling detection systems (Amersham).

For the complementation plasmid, the *mcc* cluster was cloned downstream of an *hsp60* promoter in the integrative plasmid pMV306kan using primers 1 and 2 (Table S1). This plasmid was electroporated into *Δmcc* competent cells and plated on 7H11 agar with hygromycin B and kanamycin.

4.3. Growth curves

M. tuberculosis strains were grown in stationary flasks in 7H9 and supplement with a starting optical density at 580 nm (OD_{580nm}) of 0.02–0.03 at 37 °C. OD_{580nm} measurements were taken at indicated time points.

4.4. Minimal inhibitory concentration (MIC) analysis

M. tuberculosis strains were grown to log phase and diluted to OD_{580nm} of 0.01 in 7H9 with supplement. 200 μL of cells were added to each well of a 96-well plate (Costar) and 2 μL compounds were added in a 2-fold dose response. After 7–8 days of incubation at 37 °C, OD_{580nm} was read. MIC_{90} is defined as the lowest concentration of compound that gave 90% inhibition of growth.

4.5. Nutrient deprivation experiments

M. tuberculosis strains were grown to mid-late log phase, washed twice with Dulbecco PBS/0.02% tyloxapol and spun at 120 × *g* for 8 min without break to yield a single cell suspension. Cells were diluted to 5×10^7 CFU/mL in PBS/0.02% tyloxapol and left in stationary flasks at 37 °C for six weeks. At six weeks, samples were diluted 10-fold, plated on 7H11 plates, incubated at 37 °C for 21 days and colonies were then counted.

4.6. Clofazimine CFU

M. tuberculosis strains were grown to log phase, washed once with Dulbecco PBS/0.02% tyloxapol and spun at 120 × *g* for 8 min without break to yield a single cell suspension. Cells were diluted to OD_{580nm} of 0.01 in 7H9 with supplement. 200 μL of each strain were added in triplicate to a 96-well plate (Costar) and 2 μL clofazimine (Sigma) stock or DMSO vehicle were added. After incubation for indicated time, samples were diluted 10-fold, plated on 7H11 plates, incubated at 37 °C for 21 days and colonies were then counted. Clofazimine-treated *Δmcc* colonies were counted a few days later.

4.7. Peroxide CFU

M. tuberculosis strains were grown to log phase, washed once with Dulbecco PBS/0.02% tyloxapol and spun at 120 × *g* for 8 min without break to yield a single cell suspension. Cells were diluted to OD_{580nm} of 0.1 in 7H9 with supplement. 500 μL of each strain were added in triplicate to a 24-well plate (Costar) and 5.5 mM H_2O_2 (Sigma) or water were added. After 4 h, samples were diluted 10-fold, plated on 7H10 plates, incubated at 37 °C for 21 days and colonies were then counted.

Acknowledgements

The authors thank Drs. Selin Somersan-Karakaya, Julien Vaubourgeix, Ben Gold, Carl Nathan and members of the Carl Nathan lab at Weill Cornell Medicine for useful discussions. We thank Dr. David Zhang for data analysis. We thank Drs. Julien Vaubourgeix and Carl Nathan for critical review of this manuscript. pNIT(kan)::RecET-SacB was a gift from Drs. Christopher Sassetti and Kenan Murphy (University of Massachusetts).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.02.002.

Funding

This work was funded by the Abby and Howard P. Milstein Program in Chemical Biology and Translational Medicine and the Tri-Institutional TB Research Unit (NIH Grant U19 AI111143).

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