AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

Susceptibility of *Mycobacterium tuberculosis* Cytochrome *bd* Oxidase Mutants to Compounds Targeting the Terminal Respiratory Oxidase, Cytochrome *c*

Atica Moosa,[a] Dirk A. Lamprecht,[b] Kriti Arora,[c] Clifton E. Barry III,[c,d] Helena I. M. Boshoff,[c] Thomas R. Ioerger,[e] Adrie J. C. Steyn,[b,f,g] Valerie Mizrahi,[a,d] Digby F. Warner[a,d]

MRC/NHLS/UCT Molecular Mycobacteriology Research Unit, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, Department of Pathology, University of Cape Town, Cape Town, South Africa[a]; Africa Health Research Institute, Durban, South Africa[b]; Tuberculosis Research Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA[c]; Institute of Infectious Disease & Molecular Medicine, University of Cape Town, Cape Town, South Africa[d]; Department of Computer Science and Engineering, Texas A&M University, College Station, Texas, USA[e]; Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama, USA[f]; Centres for AIDS Research and Free Radical Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA[g]

ABSTRACT We deleted subunits I (*cydA*) and II (*cydB*) of the *Mycobacterium tuberculosis* cytochrome *bd* menaquinol oxidase. The resulting Δ*cydA* and Δ*cydAB* mutants were hypersusceptible to compounds targeting the mycobacterial bc_1 menaquinol-cytochrome *c* oxidoreductase and exhibited bioenergetic profiles indistinguishable from strains deficient in the ABC-type transporter, CydDC, predicted to be essential for cytochrome *bd* assembly. These results confirm CydAB and CydDC as potential targets for drugs aimed at inhibiting a terminal respiratory oxidase implicated in pathogenesis.

KEYWORDS TB drug discovery, mycobacterial respiration, electron transport chain, extracellular flux analysis, oxidative phosphorylation

There is resurgent interest in mycobacterial respiration and energy metabolism as potential sources of new targets and improved compounds for tuberculosis (TB) chemotherapy (1–3). This has been fueled primarily by the success of bedaquiline (BDQ), a diarylquinoline that inhibits the mycobacterial ATP synthase (4) and is approved for clinical use against multidrug-resistant (MDR) TB (5). However, additional agents in the TB drug discovery pipeline include Q203, an imidazopyridine that targets the mycobacterial cytochrome bc_1 complex (6), as well as the repurposed drug, clofazimine, which acts via a redox cycling mechanism involving reduction by the type II NADH dehydrogenase followed by nonenzymatic oxidation that produces reactive oxygen species (7). Moreover, a number of recent studies have demonstrated the potential to inhibit other components of the mycobacterial electron transport chain (ETC) (1, 2, 8) as well as the opportunities inherent in simultaneously targeting multiple components of mycobacterial oxidative phosphorylation (3, 9).

The rationale is strong: respiration is essential for the survival of replicating and nonreplicating bacilli (10). In addition, while the flexibility inherent in the multiply branched mycobacterial electron transport chain implies redundancy (8), the dependence of *Mycobacterium tuberculosis* on a single lipoquinone, menaquinone, and only two terminal respiratory oxidases—the aa_3-type cytochrome *c* oxidase and the cyto-

Received 28 June 2017 **Accepted** 22 July 2017

Accepted manuscript posted online 31 July 2017

Citation Moosa A, Lamprecht DA, Arora K, Barry CE, III, Boshoff HIM, Ioerger TR, Steyn AJC, Mizrahi V, Warner DF. 2017. Susceptibility of *Mycobacterium tuberculosis* cytochrome *bd* oxidase mutants to compounds targeting the terminal respiratory oxidase, cytochrome *c*. Antimicrob Agents Chemother 61:e01338-17. https://doi.org/10.1128/AAC.01338-17.

Address correspondence to Digby F. Warner, digby.warner@uct.ac.za.

chrome *bd* menaquinol oxidase (11)—suggests the potential for targeted disruption of respiratory function for both adjunctive (12) and combination (3) strategies. The *cydAB*-encoded cytochrome *bd* functions as the terminal acceptor in *M. tuberculosis* under microaerophilic conditions (11) and is also able to support aerobic growth during chemical inhibition of the bc_1 complex, QcrCAB; for example, following exposure to imidazopyridines (3, 13).

In *Escherichia coli*, assembly of cytochrome *bd* is dependent on the ABC-type transporter, CydDC, which is also required for the synthesis of other periplasmic cytochromes (14). As a result, *cydAB* and *cydDC* mutants of *E. coli* exhibit overlapping, but distinct, phenotypes, consistent with the genomic separation of the two operons. In contrast, the *cydDC* genes in *M. tuberculosis* are operonic with *cydAB*. At the inception of this study, it was not known whether *M. tuberculosis* CydDC functioned solely in cytochrome *bd* biosynthesis; previous reports exploited a *cydC*::*aph* mutant (15), in which only the terminal gene of the *cydABDC* locus was eliminated, or a Δ*cydA*::*hyg* mutant (12), which was expected to disrupt full operon function owing to polar effects. There were also two articles that utilized a knockout mutant, the "cydKO" strain, reportedly lacking the 3′ end of *cydB*, the entire *cydD*, and the 5′ end of *cydC* (9, 13); however, a subsequent author correction to reference 13 has noted that the strain actually employed in those papers was the *cydC*::*aph* mutant (15). It was not clear, therefore, when we initiated the current study whether disruption of the entire locus was phenotypically equivalent to targeted deletion (or, by implication, chemical inhibition) of the individual genes; moreover, no reports at the time had attempted to unlink the effects of disrupted cytochrome *bd* menaquinol oxidase function (*cydAB* inactivation) from deficient ABC transport (*cydDC* inactivation).

In a key study published during the preparation of the manuscript, Berney, Pethe, and colleagues (3) reported that targeted disruption of *cydAB* eliminated oxygen respiration in *M. tuberculosis* bacilli exposed to Q203, killing the cells and rendering the resulting Δ*cydAB* mutant strain hypersusceptible to Q203 treatment *in vitro* in both replicating and nonreplicating (tolerant) conditions as well as in a mouse model. In that case, Δ*cydAB* mutants were constructed using a phage-mediated unmarking (16) system that leaves an approximately 130-bp "scar" at the deletion site following activity of the γδ resolvase.

Here, we generated targeted, in-frame deletion mutants of *cydA* and *cydAB* in *M. tuberculosis* H37RvMA (17) using two-step allelic exchange mutagenesis (18) in order to preserve the sequence integrity of the locus. This was confirmed by PCR and whole-genome sequencing (see Table S1 in the supplemental material). In standard microplate-based alamarBlue assays (MABA) (19) using Middlebrook 7H9 liquid growth medium (Difco) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 0.5% glycerol, and 0.05% Tween 80, the MIC_{90} values recorded for a representative panel of approved anti-TB agents from different antibiotic classes and with diverse mechanisms of action were the same for *cydA* and *cydAB* mutants and identical to those observed for wild-type *M. tuberculosis* H37Rv and the cydKO (*cydC*::*aph*) mutant strain (Table 1). That is, no hypersensitivity phenotype was observed for any of the *cyd* mutants against any of the agents tested. This included BDQ, which was in contrast to some reports (12) but consistent with other recent results (3, 9). All three *cyd* mutants were, however, hypersusceptible to experimental compounds for which resistance maps to *qcrB* (Table 1; see also Fig. S1 in the supplemental material). Moreover, there were no significant differences in MIC values across the *cydA*, *cydAB*, and cydKO (*cydC*::*aph*) strains, suggesting that elimination of either the CydAB oxidase or CydDC transport subunits was sufficient to abrogate cytochrome *bd* function.

To investigate the impact of the different *cyd* alleles on mycobacterial respiratory function, we determined the bioenergetic responses of the cydKO (*cydC*::*aph*), *cydA*, and *cydAB* strains to Q203 treatment. Previously, we showed that Q203 inhibits electron flux through the *M. tuberculosis* cytochrome bc_1, and this block is alleviated by rerouting electrons through cytochrome *bd* (9). Moreover, to compensate for the decreased proton motive force (PMF) generated by cytochrome *bd* and consequent

TABLE 1 MIC determinations against wild-type *M. tuberculosis* H37Rv and *cyd* mutant strains

Compound	*M. tuberculosis* strain[a]			
	H37Rv	cydKO (*cydC*::*aph*) mutant strain	Δ*cydA* mutant strain	Δ*cydAB* mutant strain
Rifampin	0.01	0.01	0.01	0.01
Isoniazid	0.04	0.04	0.04	0.04
Streptomycin	0.9	0.9	0.9	0.9
Ethambutol	0.47	0.47	0.47	0.47
Pretomanid (PA-824)	0.1	0.1	0.1	0.1
Levofloxacin	0.94	0.94	0.94	0.94
BDQ	0.03	0.03–0.06	0.03	0.03–0.06
Q203	0.0097 (>50)[c]	0.0003	0.0012	0.0003
Compound 1[b]	3.125 (>50)[c]	0.39	0.39	0.39
Compound 2[b]	0.390 (>25)[c]	0.02	0.0488	0.02

[a]All values are 14-day MABA (19) MIC_{90}s and are reported in micrograms per milliliter.
[b]Compound numbers are as per reference 13.
[c]The MIC was determined visually according to the presence/absence of a definite mycobacterial pellet.

drop in ATP production, bacilli increase the total electron flux through the alternative terminal oxidase, with a subsequent increase in oxygen consumption rate (OCR) (9). In contrast, in the cydKO mutant strain, Q203 treatment is associated with a rapid decrease in OCR owing to the complete inhibition of terminal oxidase function.

As observed previously (9), exposure of wild-type *M. tuberculosis* H37Rv to Q203 caused a significant increase in the OCR from basal levels (measurement ten [M10]) (Fig. 1A). In contrast, OCR decreased in the cydKO (*cydC*::*aph*) (M10 in Fig. 1B) strain, an effect which was also observed in the *cydA* and *cydAB* strains (M10 in Fig. 1C and

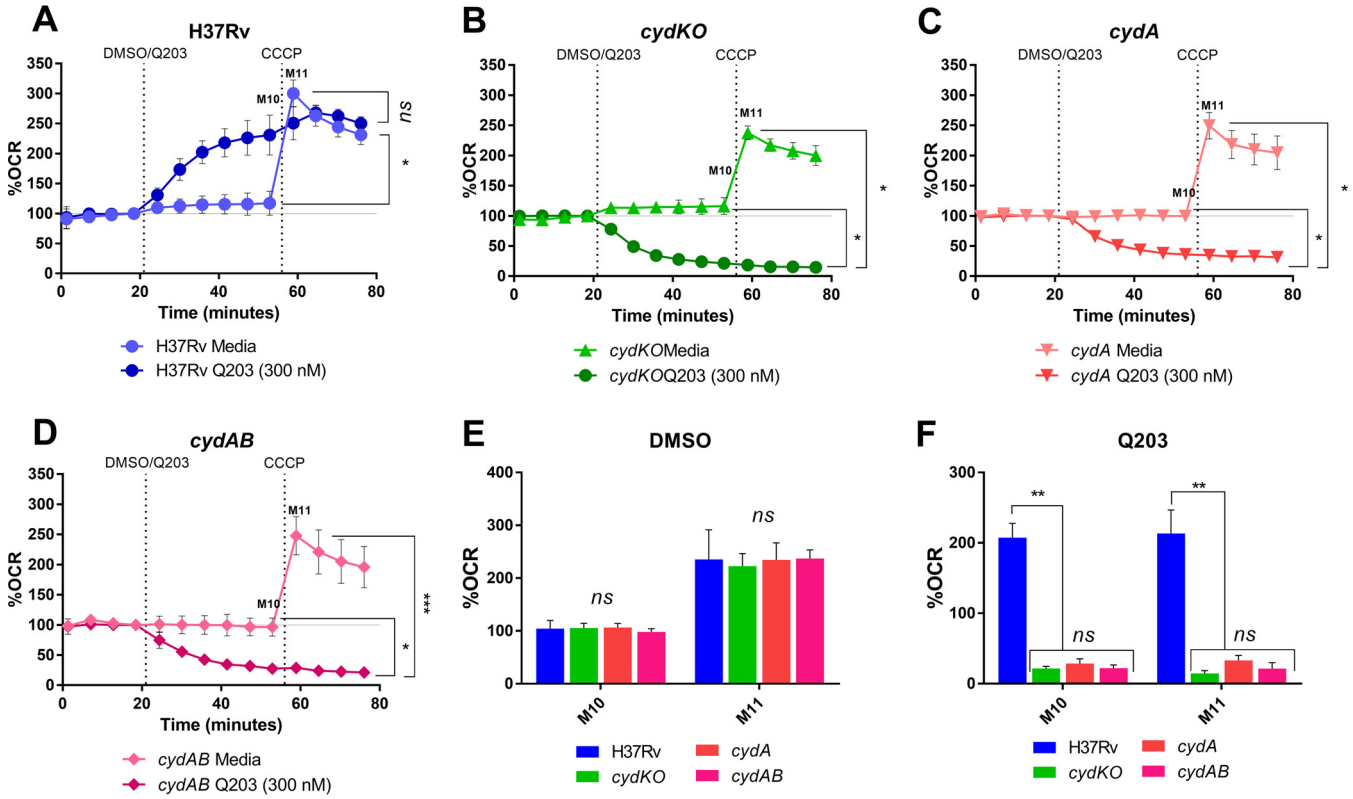

FIG 1 The *cyd* operon mutants are characterized by near-identical bioenergetics profiles. (A to D) The OCR profiles of wild-type *M. tuberculosis* H37RvMA and the cydKO (*cydC*::*aph*), Δ*cydA*, and Δ*cydAB* mutant strains treated with 300 nM Q203 and DMSO (as vehicle control), respectively. All plots are representative of three independent experiments, and the statistical analysis was by analysis of variance (ANOVA) (95% confidence interval) for the three biological replicates. ns, not significant; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (ANOVA, GraphPad Prism 6.05).

D) and was consistent with the phenotype of the Δ*cydAB* mutant described by Berney, Pethe, and colleagues (3). Following addition of the protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), there was no increase in OCR in any of the Q203-treated *cyd* mutants (M11 in Fig. 1B to D); this was in contrast to that in wild-type *M. tuberculosis* H37Rv, as well as that in the mock (dimethyl sulfoxide [DMSO])-treated *cyd* mutants (M11 in Fig. 1A to D), and indicated the complete shutdown of electron flux through the ETC in the absence of functional cytochrome *bd*. No significant differences were detected in OCR levels (M10 and M11) of all strains—wild-type strain and *cyd* mutants—treated with DMSO (Fig. 1E). This indicated that, in the absence of Q203 treatment, ETC function of the *cyd* deletion mutants was similar to that of the wild type; basal OCR levels were the same (M10), and all strains exhibited comparable capacity to raise OCR to maintain membrane potential upon uncoupling through CCCP addition (M11). Moreover, the OCR levels of the Q203-treated *cyd* strains were equivalent, and all three mutants exhibited the same inability to elevate OCR levels after CCCP exposure (Fig. 1F).

In combination, our results indicate that disruption of any of the *cyd* operon genes (or any combination thereof) results in a cytochrome *bd* functionally deficient mutant characterized by a common, but distinct, bioenergetic profile that is consistent with the observed hypersusceptibility to compounds inhibiting the cytochrome *c* respiratory oxidase. As such, these observations offer support to recent work which has provided compelling evidence of the potential for pathway-specific combination therapies to cripple metabolic escape mechanisms, thereby enhancing compound cidality and eliminating drug-tolerant bacilli (3).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01338-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

We thank Vinayak Singh for technical assistance.

This work was supported by the Strategic Health Innovation Partnerships (SHIP) initiative of the South African Medical Research Council (to D.F.W. and A.J.C.S.), the South African Medical Research Council (to V.M.), the National Research Foundation of South Africa (to V.M.), the Intramural Research Program of the NIAID, NIH (to C.E.B.), and the Foundation for the National Institutes of Health with support from the Bill & Melinda Gates Foundation (to C.E.B. and V.M.).

We declare no conflicts of interest.

REFERENCES

1. Bald D, Villellas C, Lu P, Koul A. 2017. Targeting energy metabolism in *Mycobacterium tuberculosis*, a new paradigm in antimycobacterial drug discovery. mBio 8:e00272-17. https://doi.org/10.1128/mBio.00272-17.
2. Sukheja P, Kumar P, Mittal N, Li SG, Singleton E, Russo R, Perryman AL, Shrestha R, Awasthi D, Husain S, Soteropoulos P, Brukh R, Connell N, Freundlich JS, Alland D. 2017. A novel small-molecule inhibitor of the *Mycobacterium tuberculosis* demethylmenaquinone methyltransferase MenG is bactericidal to both growing and nutritionally deprived persister cells. mBio 8:e02022-16. https://doi.org/10.1128/mBio.02022-16.
3. Kalia NP, Hasenoehrl EJ, Ab Rahman NB, Koh VH, Ang MLT, Sajorda DR, Hards K, Grüber G, Alonso S, Cook GM, Berney M, Pethe K. 2017. Exploiting the synthetic lethality between terminal respiratory oxidases to kill *Mycobacterium tuberculosis* and clear host infection. Proc Natl Acad Sci U S A 114:7426–7431. https://doi.org/10.1073/pnas.1706139114.
4. Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V. 2005. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science 307:223–227. https://doi.org/10.1126/science.1106753.
5. Dheda K, Gumbo T, Maartens G, Dooley KE, McNerney R, Murray M, Furin J, Nardell EA, London L, Lessem E, Theron G, van Helden P, Niemann S, Merker M, Dowdy D, Van Rie A, Siu GK, Pasipanodya JG, Rodrigues C, Clark TG, Sirgel FA, Esmail A, Lin HH, Atre SR, Schaaf HS, Chang KC, Lange C, Nahid P, Udwadia ZF, Horsburgh CR, Jr, Churchyard GJ, Menzies D, Hesseling AC, Nuermberger E, McIlleron H, Fennelly KP, Goemaere E, Jaramillo E, Low M, Jara CM, Padayatchi N, Warren RM. 2017. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. Lancet Respir Med 5:291–360. https://doi.org/10.1016/S2213-2600(17)30079-6.
6. Pethe K, Bifani P, Jang J, Kang S, Park S, Ahn S, Jiricek J, Jung J, Jeon HK, Cechetto J, Christophe T, Lee H, Kempf M, Jackson M, Lenaerts AJ, Pham H, Jones V, Seo MJ, Kim YM, Seo M, Seo JJ, Park D, Ko Y, Choi I, Kim R, Kim SY, Lim S, Yim SA, Nam J, Kang H, Kwon H, Oh CT, Cho Y, Jang Y, Kim J, Chua A, Tan BH, Nanjundappa MB, Rao SP, Barnes WS, Wintjens R, Walker JR, Alonso S, Lee S, Kim J, Oh S, Oh T, Nehrbass U, Han SJ, No Z, et al. 2013. Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. Nat Med 19:1157–1160. https://doi.org/10.1038/nm.3262.

7. Yano T, Kassovska-Bratinova S, Teh JS, Winkler J, Sullivan K, Isaacs A, Schechter NM, Rubin H. 2011. Reduction of clofazimine by mycobacterial type 2 NADH:quinone oxidoreductase: a pathway for the generation of bactericidal levels of reactive oxygen species. J Biol Chem 286:10276–10287. https://doi.org/10.1074/jbc.M110.200501.
8. Black PA, Warren RM, Louw GE, van Helden PD, Victor TC, Kana BD. 2014. Energy metabolism and drug efflux in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 58:2491–2503. https://doi.org/10.1128/AAC.02293-13.
9. Lamprecht DA, Finin PM, Rahman MA, Cumming BM, Russell SL, Jonnala SR, Adamson JH, Steyn AJ. 2016. Turning the respiratory flexibility of *Mycobacterium tuberculosis* against itself. Nat Commun 7:12393. https://doi.org/10.1038/ncomms12393.
10. Boshoff HI, Barry CE, III. 2005. Tuberculosis-metabolism and respiration in the absence of growth. Nat Rev Microbiol 3:70–80. https://doi.org/10.1038/nrmicro1065.
11. Cook GM, Hards K, Vilcheze C, Hartman T, Berney M. 2014. Energetics of respiration and oxidative phosphorylation in mycobacteria. Microbiol Spectr 2. https://doi.org/10.1128/microbiolspec.MGM2-0015-2013.
12. Berney M, Hartman TE, Jacobs WR, Jr. 2014. A *Mycobacterium tuberculosis* cytochrome *bd* oxidase mutant is hypersensitive to bedaquiline. mBio 5:e01275-14. https://doi.org/10.1128/mBio.01275-14.
13. Arora K, Ochoa-Montano B, Tsang PS, Blundell TL, Dawes SS, Mizrahi V, Bayliss T, Mackenzie CJ, Cleghorn LA, Ray PC, Wyatt PG, Uh E, Lee J, Barry CE, III, Boshoff HI. 2014. Respiratory flexibility in response to inhibition of cytochrome *c* oxidase in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 58:6962–6965. https://doi.org/10.1128/AAC.03486-14.
14. Cook GM, Cruz-Ramos H, Moir AJ, Poole RK. 2002. A novel haem compound accumulated in *Escherichia coli* overexpressing the *cydDC* operon, encoding an ABC-type transporter required for cytochrome assembly. Arch Microbiol 178:358–369. https://doi.org/10.1007/s00203-002-0467-6.
15. Shi L, Sohaskey CD, Kana BD, Dawes S, North RJ, Mizrahi V, Gennaro ML. 2005. Changes in energy metabolism of *Mycobacterium tuberculosis* in mouse lung and under *in vitro* conditions affecting aerobic respiration. Proc Natl Acad Sci U S A 102:15629–15634. https://doi.org/10.1073/pnas.0507850102.
16. Jain P, Hsu T, Arai M, Biermann K, Thaler DS, Nguyen A, Gonzalez PA, Tufariello JM, Kriakov J, Chen B, Larsen MH, Jacobs WR, Jr. 2014. Specialized transduction designed for precise high-throughput unmarked deletions in *Mycobacterium tuberculosis*. mBio 5:e01245-14. https://doi.org/10.1128/mBio.01245-14.
17. Ioerger TR, Feng Y, Ganesula K, Chen X, Dobos KM, Fortune S, Jacobs WR, Jr, Mizrahi V, Parish T, Rubin E, Sassetti C, Sacchettini JC. 2010. Variation among genome sequences of H37Rv strains of *Mycobacterium tuberculosis* from multiple laboratories. J Bacteriol 192:3645–3653. https://doi.org/10.1128/JB.00166-10.
18. Gopinath K, Warner DF, Mizrahi V. 2015. Targeted gene knockout and essentiality testing by homologous recombination. Methods Mol Biol 1285:131–149. https://doi.org/10.1007/978-1-4939-2450-9_8.
19. Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH. 1998. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J Clin Microbiol 36:362–366.