JOURNAL OF BACTERIOLOGY, Sept. 2005, p. 6300–6308 Vol. 187, No. 18
0021-9193/05/$08.00+0 doi:10.1128/JB.187.18.6300–6308.2005

Function of the Cytochrome bc_1-aa_3 Branch of the Respiratory Network in Mycobacteria and Network Adaptation Occurring in Response to Its Disruption†

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Received 29 April 2005/Accepted 15 June 2005

The aerobic electron transport chain in *Mycobacterium smegmatis* can terminate in one of three possible terminal oxidase complexes. The structure and function of the electron transport pathway leading from the menaquinol-menaquinone pool to the cytochrome bc_1 complex and terminating in the aa_3-type cytochrome *c* oxidase was characterized. *M. smegmatis* strains with mutations in the bc_1 complex and in subunit II of cyctochome *c* oxidase were found to be profoundly growth impaired, confirming the importance of this respiratory pathway for mycobacterial growth under aerobic conditions. Disruption of this pathway resulted in an adaptation of the respiratory network that is characterized by a marked up-regulation of *cydAB*, which encodes the bioenergetically less efficient and microaerobically induced cytochrome *bd*-type menaquinol oxidase that is required for the growth of *M. smegmatis* under O_2-limiting conditions. Further insights into the adaptation of this organism to rerouting of the electron flux through the branch terminating in the *bd*-type oxidase were revealed by expression profiling of the bc_1-deficient mutant strain using a partial-genome microarray of *M. smegmatis* that is enriched in essential genes. Although the expression profile was indicative of an increase in the reduced state of the respiratory chain, blockage of the bc_1-aa_3 pathway did not induce the sentinel genes of *M. smegmatis* that are induced by oxygen starvation and are regulated by the DosR two-component regulator.

The ability of *Mycobacterium tuberculosis* to grow and persist in its human host and to establish and maintain a state of latent tuberculosis infection from which it can reactivate to cause disease is critical to its extraordinary success as a pathogen. This remarkable competence is attributable to *M. tuberculosis*'s ability to adapt physiologically to the environmental conditions encountered during the course of an infection. These conditions are thought to include restricted nutrient availability and oxidative and nitrosative stress imposed by the host immune response (10, 37, 39, 41).

Of these, the mechanism of physiological adaptation to O_2 restriction has been most intensively investigated. These studies have revealed that gradual depletion of O_2 from cultures of *M. tuberculosis* leads to progression of this organism through two stages of nonreplicating persistence, confirming that its replication requires the availability of O_2 (41). Of particular importance was the observation that a rapid shift from an aerobic environment to an oxygen-deficient one resulted in bacterial death, indicating that ordered metabolic shutdown is necessary for adaptation to hypoxia or anoxia. Recent studies that have utilized transcriptional profiling to explore the metabolic changes that occur in *M. tuberculosis* in response to inhibiting aerobic electron flow by O_2 depletion (5, 23, 31, 39) and by other means (5, 39) have revealed intriguing insights into the respiratory network of *M. tuberculosis* that underscore the need to investigate its function and regulation at a molecular level (6).

Mycobacteria possess a branched aerobic respiratory chain in which electrons flow from NADH dehydrogenase and succinate dehydrogenase complexes into the menaquinone-menaquinol pool, from where they are transferred either to an aa_3-type cytochrome *c* oxidase (CcO) via the cytochrome bc_1 complex or directly to the cytochrome *bd*-type menaquinol oxidase (6, 16, 42). The branch terminating in the *bd*-type oxidase was shown to be important for microaerobic respiration in *M. smegmatis* (16) and as such is functionally analogous to *bd*-type oxidase-terminating branches in other organisms (25). In contrast, little is currently known about the CcO-terminating branch in mycobacteria. Genome comparisons suggest that its structure resembles those of other nocardio-

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† Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
M. tuberculosis H37Rv	Laboratory strain ATCC 27294	Laboratory collection
M. smegmatis		
mc^{2}155	High-frequency transformation mutant of ATCC 607	34
Δ*qcrCAB*::*hyg*	*qcrCAB* deletion-replacement mutant of mc^{2}155; Hyg^r	This work
Δ*ctaC*::*hyg*	*ctaC* deletion-replacement mutant of mc^{2}155; Hyg^r	This work
ctaDI$^{+/-}$	Derivative of mc^{2}155 with one wild-type *ctaDI* and one Δ*ctaD*::*hyg* allele	This work
Δ*ctaDII*::*hyg*	*ctaDII* deletion-replacement mutant of mc^{2}155; Hyg^r	This work
mc^{2}155::pBK4	mc^{2}155 carrying pBK4 integrated at *cyd* locus; Km^r	16
Δ*qcrCAB*::*hyg*::pBK4	Δ*qcrCAB*::*hyg* carrying pBK4 integrated at *cyd* locus; Hyg^r Km^r	This work
Plasmids		
pGEM3Z(+)f	*E. coli* cloning vector; Ap^r	Promega
pGEMTeasy	*E. coli* cloning vector for cloning PCR products; Ap^r	Promega
pCR2.1-TOPO	*E. coli* vector for cloning PCR products; Km^r Ap^r	Invitrogen
p2NIL	*E. coli* vector for cloning homologous recombination substrates; Km^r	22
pGOAL17	Vector carrying P_{Ag85}-*lacZ* P_{hsp60}-*sacB* PacI marker cassette; Ap^r	22
pIJ963	*E. coli* vector carrying *hyg* cassette	2
pBK4	p2NIL containing the *M. smegmatis cydA′*::*lacZ* fusion cassette; Hyg^r	16
pQCRTBKO	Knockout vector carrying *M. tuberculosis* Δ*qcrCAB* allele; Hyg^r Km^r	This work
pCTACTBKO	Knockout vector carrying *M. tuberculosis* Δ*ctaC*::*hyg* allele; Hyg^r Km^r	This work
pQCRSMKO	Knockout vector carrying *M. smegmatis* Δ*qcrCAB*::*hyg* allele; Hyg^r Km^r	This work
pCTADISMKO	Knockout vector carrying *M. smegmatis* Δ*ctaDI*::*hyg* allele; Hyg^r Km^r	This work
pCTADIISMKO	Knockout vector carrying *M. smegmatis* Δ*ctaDII*::*hyg* allele; Hyg^r Km^r	This work
pCTACSMKO	Knockout vector carrying *M. smegmatis* Δ*ctaC*::*hyg* allele; Hyg^r Km^r	This work

[a] Ap^r, ampicillin resistance; Hyg^r, hygromycin resistance; Km^r, kanamycin resistance.

form actinomycetes, such as *Corynebacterium glutamicum* (7, 18, 19, 29, 35) and *Rhodococcus rhodochrous* (36), which are distinguished by the fact that the cytochrome c_1 (QcrC) in these organisms is a distinct, diheme *c*-type cytochrome (18, 35).

A resulting feature of note inferred from studies in *C. glutamicum* (7, 19) is that the mycobacterial bc_1 complex and the cytochrome aa_3 oxidase would be expected to form a supercomplex. We have adopted a genetic approach to investigate the function of the bc_1-aa_3 branch in mycobacteria and, in this paper, report the construction of mutations in *M. smegmatis* genes encoding the bc_1 complex and the aa_3-type CcO. The results of this study demonstrate the importance of the cytochrome bc_1-aa_3 branch for mycobacterial growth and suggest that the disruption of this pathway is accompanied by an adaptation of the respiratory network that is characterized by constitutive up-regulation of the menaquinol oxidase, cytochrome *bd*-type menaquinol oxidase, and some genes previously shown to be induced by hypoxia, such as *uspL* (5, 20, 23, 31) and Rv1592c (5, 23, 31), but not of sentinel, DosR-regulated genes (20).

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are shown in Table 1. The vectors pQCRTBKO and pCTACTBKO were used for knockout of *qcrCAB* and *ctaC*, respectively, in *M. tuberculosis*, whereas pQCRSMKO, pCTADSMKO, pCATD2SMKO, and pCATCSMKO were used for knockout of the *qcrCAB*, *ctaDI*, *ctaDII*, and *ctaC* genes, respectively, in *M. smegmatis*. A deletion allele of *M. tuberculosis qcrCAB* was constructed by ligating the 3,147-bp upstream ScaI/PstI fragment (containing the 5′-terminal 240 bp of *qcrC*) to the 3,256-bp downstream PstI fragment (containing the 3′-terminal 800 bp of *qcrB*) in p2NIL (22), thus eliminating an internal, 2,734-bp region from the *qcrCAB* operon. The *hyg-lacZ-sacB* marker cassette from pGOAL19 (22) was cloned in the PacI site of the resulting plasmid to produce pQCRTBKO.

Deletion alleles of the remaining genes were constructed by PCR amplification of 5′- and 3′-flanking regions using the primer pairs shown in Table 2. In the case of the *M. smegmatis* genes, the PCR primers were designed based on the open reading frame sequences identified in the preliminary genome sequence of strain mc^{2}155 from the Institute for Genomic Research (http://www.tigr.org/ufmg/). The flanking regions were cloned in pGEMTeasy or pCR2.1-TOPO before simultaneously subcloning both fragments in p2NIL (22). The deletion alleles were marked by the insertion of a hygromycin resistance gene at the junction site before introduction of the *lacZ-sacB* marker cassette from pGOAL17 (22) in the PacI site of p2NIL (22). The knockout vectors were electroporated into *M. tuberculosis* or *M. smegmatis* and mutant selection was carried out as previously described (3, 14, 22).

Bacterial culture conditions. *Escherichia coli* DH5α used for cloning procedures was grown in Luria broth (LB) or agar (LA) containing 100 μg/ml ampicillin, 50 μg/ml kanamycin, or 200 μg/ml hygromycin where necessary. *M. smegmatis* strains were grown in LB or MADC-Tw [Middlebrook 7H9 broth (Difco) supplemented with 0.085% NaCl, 0.2% glucose, 0.2% glycerol, and 0.05% Tween 80] or on LA. *M. tuberculosis* strains were grown in Middlebrook 7H9 medium supplemented with Middlebrook ADC (Difco), 0.2% glycerol, and 0.05% Tween 80 in roller bottles or as stirred cultures. Antibiotic supplements were as follows: kanamycin, 10 μg/ml (solid medium) or 25 μg/ml (liquid medium), and hygromycin, 50 μg/ml.

For growth of cultures of wild-type and mutant strains of *M. smegmatis* in shaking flasks, starter cultures at an optical density at 600 nm (OD_{600}) of ~0.013 were prepared by diluting a preculture (OD_{600} ~2.0) in 100 ml of MADC-Tw, and incubating at 37°C with shaking (350 rpm). Optical density was monitored at 3-h intervals over a period of 42 to 60 h. Oxystatic growth of *M. smegmatis* strains was carried out in a New Brunswick Scientific Bioflow 110 fermentor in batch cultures. This system is similar to the Braun BIOSTAT B system employed by Kana et al. (16), with the exception that the rotometer allowed the air supply to be controlled from 0 to 150 ml/min, and agitation speeds ranged from 50 to 1200 rpm.

Oxystatic culturing of reporter strains was carried out as follows. The fermentor was autoclaved with 990 ml of Middlebrook 7H9 and 2 ml of glycerol. Glucose and NaCl were added as the vessel cooled down, to bring the final volume to 1 liter. The vessel was allowed to equilibrate overnight, before the dissolved-O_2 probe was calibrated. The 0% air saturation level was set by sparging the medium with 100% nitrogen and the 100% air saturation level was set by sparging with 100% compressed air. The medium was inoculated with 200 ml of a preculture (OD_{600} = 2.0) to yield a starting culture of ca. 10^7 CFU/ml. To

TABLE 2. Oligonucleotides and probes used in this study

Gene(s)	Oligonucleotide	Oligonucleotide pairs used to construct knockout vector	
		Sequence (5′–3′)[a]	Region amplified
M. smegmatis qcrCAB	QCRF1	GGGCCGGAAGCttATGGGAACGGCTGGGAAGGT	878-bp product including 114 bp of 5′ end of *qcrC*
	QCRR1	CTGCCAGACgGATCcGCAGCAGAAGCCCTGCTGACA	
	QCRF2	CGCTCACCGgGATCcCGCATGCGTCCGAGCACA	863-bp product including 104 bp of 3′ end of *qcrB*
	QCRR2	GCTGTCGGgtACCTGTGCGCTGGGCGTGGC	
M. smegmatis ctaC	CTACF1	TTCGGGACGAgGTACCGCGTCACCTCGGGTGC	1,038-bp product including 130 bp of 5′ end of *ctaC*
	CTACR1	TTGCCTCAGGGGgGATcCCCGTTGGCCAGCCGAGT	
	CTACF2	CAAGGCCTACATgGATCcGCGCAACGCCGG	1,100-bp product including 139 bp of 3′ end of *ctaC*
	CATCR2	TCACGCGCGACGAGAAGCttACCGACGCCACG	
M. smegmatis ctaDI	CTAD1F1	ACCTCGTCGgGTACCGAGGCAGGCAGCCCGGCG	883-bp product including 114 bp of 3′ end of *ctaDI*
	CTAD1R1	ACTACCCGCACATGGTCGAGCGGATcCGGGCCGA	
	CTAD1F2	TGAACAGCGCCAgGAtcCCGCCGACCAGGAAGAA	787-bp product including 69 bp of 5′ end of *ctaDI*
	CTAD1R2	AATACGACGGCTGCGCGTCCGAAgCTtCGGGCAG	
M. smegmatis ctaDII	CTAD2F1	ACCCGGAGGaaGCTtGCTGGCTGTGTCCGGCGCT	859-bp product including 217 bp of 5′ end of *ctaDII*
	CTAD2R1	AACTGCAGGCCGGGATCCGCCAACTCGGTGCGGA	
	CTAD2F2	TCACGGTCGAgGATCCGTGGGGCTACGGCAACT	844-bp product including 239 bp of 3′ end of *ctaDII*
	CTAD2R2	CGCGTTTGGgGTAccTCTCCAGATCCCAACCGA	
M. tuberculosis ctaC	TBCTACF1	GGCCATCTCGTGGAAGCttCTACCCAGGTCC	1,903-bp product including 221 bp of 3′ end of *ctaC*
	TBCTACR1	TCGTGGGCCACTGCGtCtAGATGTGTGGCAC	
	TBCTACF2	AGCATTGCTGCGAGCGCCAGCTcTaGAAGACCAC	1,994-bp product including 111 bp of 5′ end of *ctaC*
	TBCTACR2	GTACTTGGTGGTGGTACGGGTaAgCTTGGCGCCC	

[a] Restriction sites used for cloning are underlined (HindIII, BamHI, KpnI), and bases changed to introduce the site are given in lowercase letters.

produce cultures for use in microarray analyses, a total culture volume of 2 liters was used.

***cyd* expression analysis using a *lacZ* reporter.** To monitor *cyd* expression in a cytochrome bc_1-deficient background, a reporter strain was constructed by electroporation of plasmid pBK4 into the Δ*qcrCAB*::*hyg* mutant, where pBK4 is a suicide plasmid carrying a *cydA′*::*lacZ* transcriptional fusion that expresses the *lacZ* gene under the control of the *cyd* promoter (16). Single crossovers were selected on media containing kanamycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and site specificity of recombination at the *cyd* locus was confirmed by Southern blot analysis. β-Galactosidase activity assays were performed as described by Kana et al. (16). To determine statistical significance, unpaired *t* tests were performed using GraphPad Instat version 3.00 (http://www.graphpad.com).

RNA purification and labeling for DNA microarrays. RNA was extracted as described by Betts et al. (1). RNA from the control experiment (2 μg) was labeled with Cy3-dCTP (Amersham Pharmacia Biotech), whereas that from the subject (4 μg) was labeled with Cy5-dCTP using previously described methods (4).

Construction of a partial-genome amplicon array of *M. smegmatis* mc^{2}155. A partial *M. smegmatis* array of 1,822 genes was constructed to analyze the transcriptional profile of the two samples. Selection was on the basis of homology with the functional genes of *Mycobacterium leprae* (9), as established via tblastn analysis. A total of 1,327 *M. leprae* genes demonstrated homology to one or more genes in *M. smegmatis*. Preliminary sequence data were obtained from the Institute for Genomic Research website at http://www.tigr.org. In addition, 99 homologs of selected *M. tuberculosis* genes were added to the array (8). Included in this list were homologs of respiratory pathway genes (including *cydA*, *cydB*, *ctaDII*, and MSMEG5584) and of those that are hypoxically inducible (31).

PCR primers with equivalent melting temperatures were designed using PRIMER3 software to amplify internal segments of each gene ranging in size from 100 to 1,000 bp (28) (source code available at http://fokker.wi.mit.edu/primer3/). Primers were synthesized by PROLIGO Australia Pty Ltd. PCR products were produced by a MWG Biotech Roboamp 4200 thermocycler in 96-well plates using the amplification protocol of 94°C for 2 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. Amplicons were evaluated by agarose gel electrophoresis with only six primer pairs not amplifying single products of the right size. Products were purified in Millipore multiscreen 96-well filtration plates and resuspended in 50% dimethyl sulfoxide (Sigma). The microarrays were printed in triplicate on Corning Gaps II slides using a Genetic Microsystems Inc. GMS 417 Arrayer. Slides were hydrated at 100°C for 5 s, cross-linked using a Stratagene UV Stratalinker 1800, baked at 80°C for 2 h, and boiled for 2 min prior to storage. The MIAME (Minimum Information About a Microarray Experiment) compliance file can be downloaded at http://vbc.med.monash.edu.au/~powell/M.smegmatis.

Microarray hybridization and data analysis. The amplicon array slides were washed in 95°C deionized water (2 min), rinsed in 95% ethanol, and quick-dried by centrifugation (500 rpm, 5 min) prior to hybridization. The slides were prehybridized for 1 h at 42°C in a buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% bovine serum albumin, and 0.1% sodium dodecyl sulfate (SDS). Labeled probes were mixed with blocking reagents (4 μg yeast tRNA, 1.9 μg herring sperm DNA) and made up to a total of 24 μl in hybridization buffer (5× SSC, 25% formamide, 0.1% SDS). Samples were denatured at 98°C for 2 min, snap-cooled on ice, and applied to the array. Hybridization was carried out under a glass coverslip in a humidified slide chamber (Corning) submerged in a 42°C water bath for approximately 16 h. Coverslips were removed in wash buffer I (1× SSC, 0.05% SDS) and slides were washed once in buffer I and twice in buffer II (0.1× SSC) for 2 min each at room temperature before being dried by centrifugation (500 rpm, 5 min). Slides were scanned using a GenePix 4000B instrument (Axon Instruments) and the resulting images were analyzed using GenePix Pro 4.0 software (Axon Instruments). Each experiment was performed using three biological replicates. Since each amplicon was spotted in triplicate on the array, a total of nine data points were obtained for each open reading frame.

Image analysis and quantification were performed using Imagene 6.0 (BioDiscovery). Spots found to be poor were flagged and removed from the analysis. Subsequent analysis was done using Bioconductor (11) and Limma (32). Spot intensities were background corrected by subtracting the background median from the foreground mean. Any resulting nonpositive values were replaced with half the minimum of all positive corrected intensities for that array. The normalization to remove various biases involved two parts. First, each array was normalized independently using print-tip loess normalization (43). Second, diagnostic plots suggested a variation in scale between arrays, so the log-ratios were scaled in such a way that each array had the same median-absolute-deviation (MAD). The normalized data were then used to fit a linear model (43) for each gene using generalized least-squares, which takes into account the correlation between duplicate spots (33). The coefficient of the fitted model for each gene describes the inferred difference in RNA expression between wild-type and mutant cells. Empirical Bayes was then used to calculate the moderated *t*

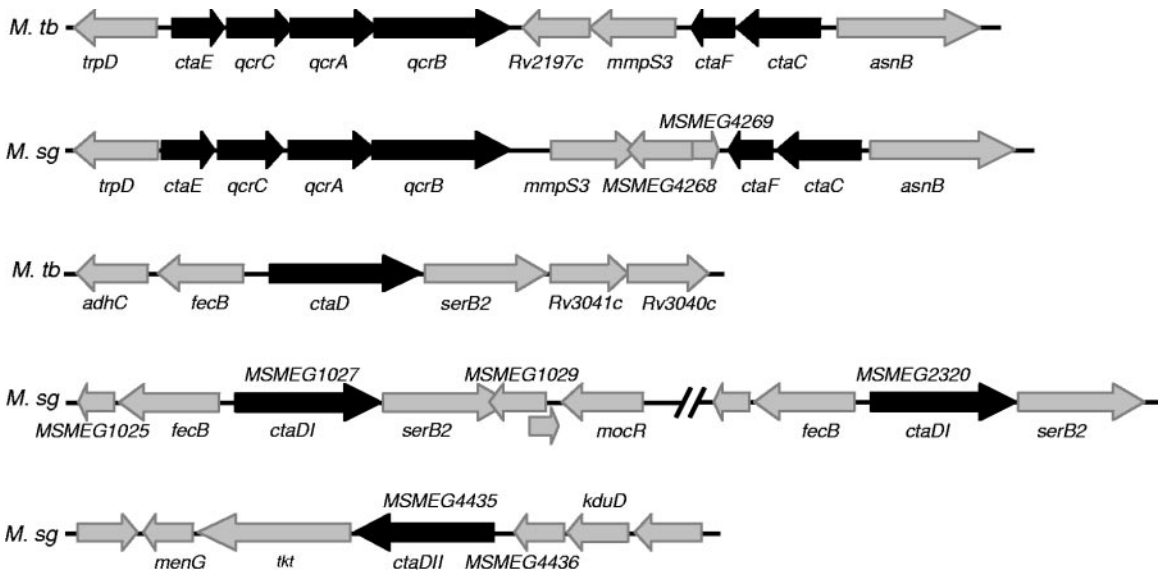

FIG. 1. Genomic organization of bc_1-aa_3 respiratory pathway genes in mycobacteria. The arrows denote the respiratory pathway (black) and neighboring genes (gray). The gene annotation for *M. tuberculosis* H37Rv is taken from Tuberculist (http://genolist.pasteur.fr/TubercuList/), and the annotation for *M. smegmatis* mc^{2}155 is from the TIGR Comprehensive Microbial Resource (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl).

statistics and associated *P* values. The *P* values were adjusted for multiple testing using false-discovery-rate. At a cutoff of 1% false-discovery-rate, 78 genes were found to be differentially expressed; this corresponds to less than one expected false-positive.

RESULTS

Targeted knockout of genes in the cytochrome bc_1-aa_3 respiratory pathway in *M. tuberculosis*. To investigate the role of the cytochrome bc_1-aa_3 branch in aerobic respiration in *M. tuberculosis*, the *qcrCAB*-encoded menaquinol-cytochrome *c* oxidoreductase was targeted for disruption by allelic exchange mutagenesis using an unmarked Δ*qcrCAB* allele delivered on a suicide plasmid (22). Both up- and downstream single crossovers were obtained. However, no double crossovers were recovered following counterselection against an upstream single crossover; all Km^s clones analyzed (20 of 20) were wild-type revertants generated by second crossover events on the same side of the deletion mutation (data not shown).

To increase the likelihood of identifying potentially growth-impairing mutations in this pathway, allelic exchange mutagenesis was attempted using a *hyg*-marked deletion-replacement allele of the *M. tuberculosis ctaC* gene, which encodes subunit II of CcO. The counterselection plates containing sucrose and hygromycin were incubated at 37°C for more than twice the normal length of time (54 versus 21 days) before colonies were picked for screening. However, no double crossovers were recovered; all clones obtained from upstream (25 of 25) and downstream single crossovers (40 of 40) were Km^r, suggesting that they were all spontaneous *sacB* mutants.

Targeted knockout of genes in the cytochrome bc_1-aa_3 pathway in *M. smegmatis*. Genes in the corresponding pathway of *M. smegmatis* were identified by BLAST searches of the unfinished genome sequence of strain mc^{2}155 (http://www.tigr.org/ufmg/) using the corresponding *M. tuberculosis* genes as query sequences. This analysis revealed the presence of three *ctaD* homologues encoding subunit I of cytochrome *c* oxidase in *M. smegmatis*, two of which are identical but differ from the third. The two identical homologues are located within a region of the genome of mc^{2}155 that is duplicated (12) and was recently shown by genome sequence analysis to be 52 kb in length and flanked by IS*1096* elements (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). This duplicated *ctaD* homologue, which is designated herein as *ctaDI*, shows 86% identity with *M. tuberculosis ctaD* at the amino acid level and is located in the same chromosomal context as *M. tuberculosis ctaD* (Fig. 1; MSMEG1027 and MSMEG2320). The second homologue, *ctaDII*, shares 85% identity with *M. tuberculosis ctaD* at the amino acid level but is located in a different chromosomal context (Fig. 1; MSMEG4435).

The duplication of *ctaDI* was confirmed by allelic exchange mutagenesis using a knockout vector carrying a Δ*ctaDI*::*hyg* allele (pCTADISMKO). Southern blot analysis of double crossovers revealed the presence of two cross-hybridizing bands of 4.6 and 2.6 kb, which correspond to the wild-type (*ctaDI*) and Δ*ctaDI*::*hyg* alleles, respectively (Fig. 2; *ctaDI*$^{+/-}$ strain). However, attempts to create a null *ctaDI* mutant of *M. smegmatis* by inactivation of the second copy of *ctaDI* in the *ctaDI*$^{+/-}$ strain failed to produce double crossovers, suggesting that this gene may be essential (data not shown). In contrast, a Δ*ctaDII*::*hyg* mutant of mc^{2}155 was readily obtained (data not shown). This strain displayed no discernible growth phenotype under aerobic conditions (data not shown), suggesting that the *ctaDII* gene is dispensable for the growth of *M. smegmatis*.

In contrast to the results obtained in *M. tuberculosis*, viable Δ*qcrCAB*::*hyg* and Δ*ctaC*::*hyg* mutants of *M. smegmatis* were obtained by allelic exchange mutagenesis (Fig. 2). However, both strains exhibited a marked reduction in growth rate in aerated liquid cultures compared to their parental wild type even though they achieved comparable cell densities in stationary phase (Fig. 3A). The growth defect of the mutant strains

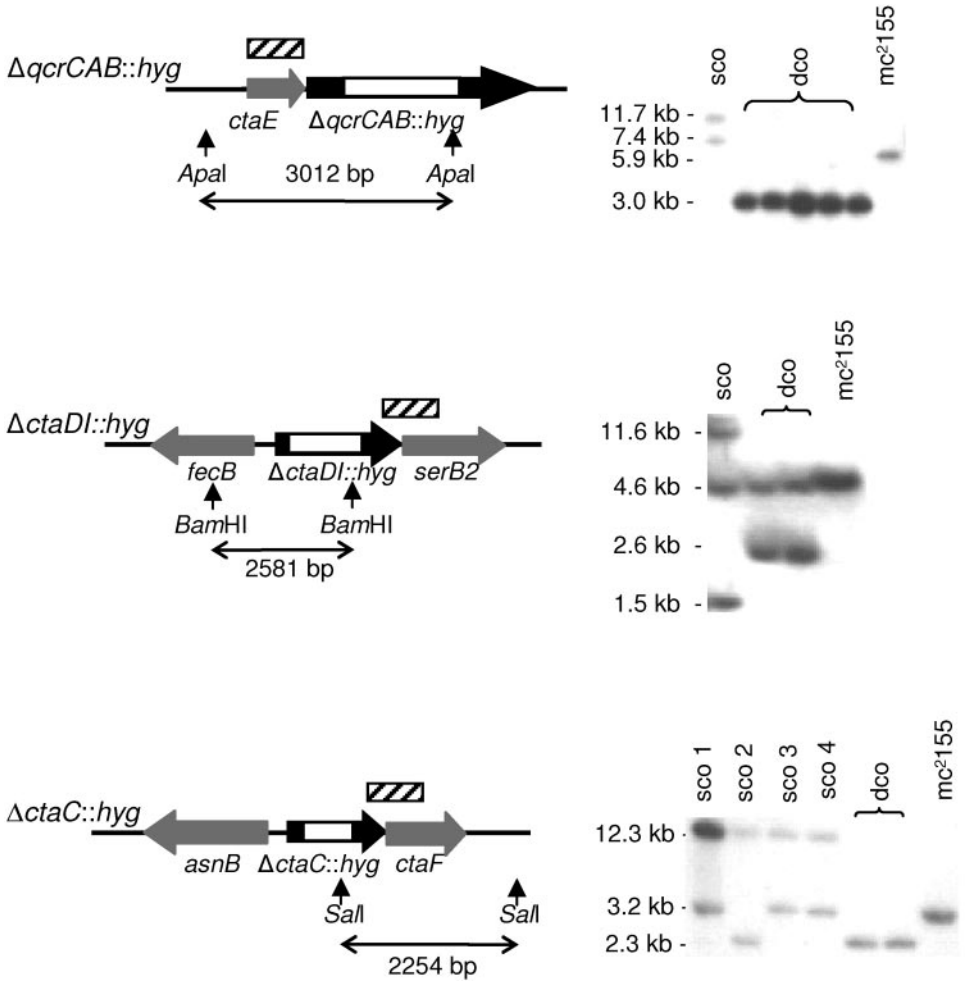

FIG. 2. Targeted knockout of bc_1-aa_3 respiratory pathway genes in *M. smegmatis* mc^{2}155. Each panel shows a schematic representation of the mutant allele. The gene(s) in which internal deletions were made is denoted by a black arrow, the *hyg* gene is denoted by a white block, and neighboring genes are shown as gray arrows. The positions of the restriction enzymes used for Southern blot analysis shown on the right of each panel are indicated by vertical arrows, and the hybridization probes used in the analysis are shown as hatched boxes above each map. Chromosomal DNA from up- and downstream single-crossover (sco) recombinants, double crossovers (dco), and the parental wild type (mc^{2}155) was digested with ApaI (*qcrCAB*), BamHI (*ctaDI*), or SalI (*ctaC*) and hybridized with the corresponding probe shown above the restriction map (hatched box).

was even more pronounced on solid medium (LA), with pinprick colonies taking a minimum of 6 days to appear (Fig. 3B). Both strains displayed visibly altered colony morphology, were highly variable in size, and continued to appear up to 2 weeks after plating.

Overexpression of cytochrome *bd* oxidase compensates for loss of the bc_1 complex. In prior work, we showed that *M. smegmatis* possesses a *cydAB*-encoded cytochrome *bd*-type menaquinol oxidase, which is induced under microaerobic conditions (16). To investigate the effect of loss of the bc_1 complex on expression of cytochrome *bd*-type menaquinol oxidase, we constructed a reporter strain that carried a *cydA′*::*lacZ* transcriptional fusion (16) site-specifically integrated at the *cyd* locus of the Δ*qcrCAB*::*hyg* mutant. Levels of β-galactosidase in the resulting Δ*qcrCAB*::*hyg*::pBK4 strain were analyzed under aerobic and microaerobic conditions and were compared with those observed in the control, mc^{2}155::pBK4, in which the same transcriptional fusion was integrated at the *cyd* locus of the parental wild type. In both strains, the *lacZ* gene is under control of the *cyd* promoter.

Induction of *cyd* gene expression was observed in wild-type *M. smegmatis* under microaerobic conditions (Fig. 4), in agreement with previous results (16). Importantly, a marked increase in expression of the *cyd* operon was observed in the Δ*qcrCAB*::*hyg* mutant over the air saturation range tested (1 to 21%). The difference in the level of *cyd* expression between the wild-type and mutant strain was significant under all conditions tested ($P < 0.0001$). However, comparison of *cyd* expression levels in the mutant strain under conditions of varying O_2 availability revealed no significant induction under microaerobic conditions above the basal expression level observed under full aeration. These results suggest that the loss of cytochrome bc_1 resulted in constitutive overproduction of the *bd*-type oxidase.

Expression profiling of the bc_1 (Δ*qcrCAB*::*hyg*) mutant. To gain further insight into the effects of rerouting aerobic elec-

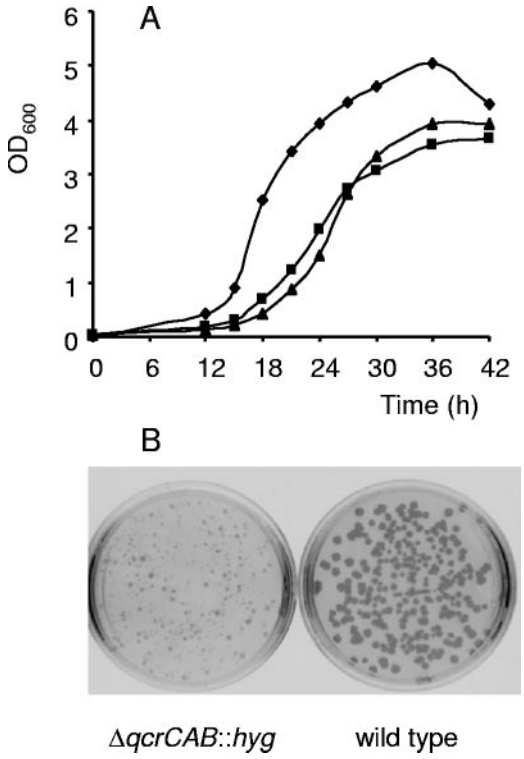

FIG. 3. Growth of the Δ*qcrCAB*::*hyg* and Δ*ctaC*::*hyg* mutants of *M. smegmatis* under aerobic conditions. (A) Growth in liquid medium. Strains were grown at 37°C in shaking flasks (350 rpm) in MADC-Tw medium, and growth was monitored by the absorbance at 600 nm (OD_{600}). ▲, Δ*qcrCAB*::*hyg*; ■, Δ*ctaC*::*hyg*; ◆, wild-type mc^2155. (B) Growth on solid medium. Strains were grown oxystatically (21% air saturation) in MADC-Tw in a bioreactor, and aliquots were withdrawn and plated on LA. Plates were incubated at 37°C for 3 days (wild type) or 6 to 8 days (mutant).

tron flow through the cytochrome *bd*-type menaquinol oxidase-terminating branch, expression profiling of aerobically grown cultures of the Δ*qcrCAB*::*hyg* mutant was carried out using a partial-genome amplicon array of *M. smegmatis*. In this experiment, Cy5-labeled cDNA from cultures of the mutant strain grown oxystatically at full aeration (21% pO_2) was hybridized against Cy3-labeled cDNA from the wild-type control cultured under identical conditions. A total of 78 genes were found to be differentially expressed, of which 42 were up- and 36 were down-regulated. The top-ranked differentially expressed genes are listed in Table 3, and the complete list is provided in the supplemental material (Table S1).

The majority of the up-regulated genes (52%) are involved in intermediary metabolism and respiration. Importantly, the presence of *cydA* in this group of differentially expressed genes was independently validated by the marked induction of *cyd* expression in the mutant strain, as deduced using the *cydA′*::*lacZ* reporter assay (Fig. 4). Other up-regulated genes included *uspL* and the *M. smegmatis* homolog of Rv1592c (MSMEG3205), which were previously shown to be induced in response to hypoxia in *M. tuberculosis* (*uspL* and Rv1592c) (5, 23, 31) and in *M. smegmatis* (*uspL*) (20). The marked up-regulation of *mihF* in the bc_1 mutant was also notable, as this gene is up-regulated in *M. smegmatis* just prior to stationary phase and may be involved in the expression of genes required for stationary-phase survival (24).

In contrast, many of the down-regulated genes are involved in information pathways (transcription and DNA repair) and in cell wall and cell processes. The most highly down-regulated gene was *lytB* (MSMEG5208). This gene is involved in the nonmevalonate pathway for the biosynthesis of terpenoids (27) and its down-regulation may thus affect the biosynthesis of menaquinone in the bc_1 mutant. The principal σ factor-encoding gene, *sigA* (13), was also markedly down-regulated in the bc_1 mutant, which is significant in light of the down-regulation of *sigA* that occurs in *M. tuberculosis* during anaerobiosis (6, 17).

DISCUSSION

Function of the cytochrome bc_1-aa_3 branch of the respiratory network in mycobacteria. A genetic approach was employed to investigate the function of the cytochrome bc_1-aa_3 branch of the respiratory network in mycobacteria. Allelic exchange mutagenesis was used to generate viable mutants of *M. smegmatis* lacking either subunit II (CtaC) of the aa_3-type CcO or the entire bc_1 complex (QcrCAB). These mutants were profoundly impaired for growth and in this respect resemble the corresponding bc_1-aa_3 pathway mutants of *C. glutamicum*, which displayed similar growth impairment in glucose minimal medium (7, 18). Together, these observations confirm that the bc_1-aa_3 pathway is the major respiratory route in actinomycetes grown under standard, aerobic culturing conditions.

Two distinct *ctaD* alleles were identified in the genome of *M. smegmatis*, *ctaDI* and *ctaDII*. CtaD is the only CcO subunit encoded by distinct alleles in *M. smegmatis*. Inactivation of *ctaDII* conferred no growth phenotype on *M. smegmatis*, confirming its dispensability for growth under the conditions tested. In contrast, a null *ctaDI* mutant lacking both copies of the *ctaDI* gene could not be recovered. The viability of other bc_1-aa_3 pathway mutants in *M. smegmatis* suggests that our

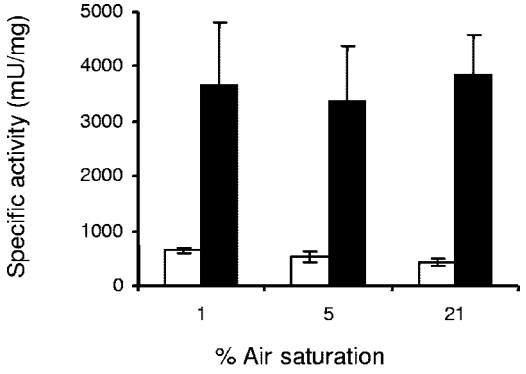

FIG. 4. Effect of cytochrome bc_1 disruption on expression of the *cyd* operon in *M. smegmatis*. Expression of the *cyd* operon was assessed using a previously described *lacZ* reporter under control of the *cyd* promoter (16) that was integrated at the *cyd* locus in the wild-type (open bars) and Δ*qcrCAB*::*hyg* (black bars) strains. The specific activity of the reporter gene product (β-galactosidase) was assessed at 21, 5, and 1% air saturation, as described under Materials and Methods. All assays were performed in duplicate and the data shown represent the averages and standard deviation of at least two independent experiments.

TABLE 3. Differentially expressed genes in aerobically grown Δ*qcrCAB*::*hyg* mutant of *M. smegmatis*

Genes and ID[a]	Gene	TIGR annotation[b]	Function[c]	M[d]	P	Class[e]
Upregulated genes						
ribA	*ribA2*	MSMEG3082	Probable riboflavin biosynthesis protein	−2.301	0.0020	7
ML2274		MSMEG3498	Probable conserved secreted protein	−1.914	0.0072	3
mihF	*mihF*	MSMEG3063	Putative integration host factor	−1.764	0.0003	2
glgC	*glgC*	MSMEG5067	Glucose-1-phosphate adenyl-transferase	−1.753	0.0072	7
ML1835		MSMEG6527	CHP	−1.556	0.0030	10
ML0510		MSMEG3035	CHP	−1.444	0.0004	10
ML0886		MSMEG4257	Possible glycosyltransferase	−1.430	0.0024	7
ML1312		MSMEG4598	CHP	−1.229	0.0001	10
argD-g	*argD*	MSMEG2446	Probable acetylornithine aminotransferase	−1.027	0.0033	7
gabD-q	*gabD*	MSMEG2554	Probable aldehyde dehydrogenase (NAD dependent)	−1.005	0.0052	7
Rv3134c	*uspL*	MSMEG5230	Universal stress protein	−0.990	0.0098	10
Rv1623c	*cydA*	MSMEG3243	Cytochrome *bd* oxidase subunit I	−0.931	0.0069	7
ML1926		MSMEG0828	Putative tuberculin-related protein	−0.915	0.0058	3
Rv1592c		MSMEG3205	CHP	−0.878	0.0016	10
Downregulated genes						
lytB2	*lytB*	MSMEG5208	Probable LytB-related protein, LytB2	3.316	0.0002	3
xseA	*xseA*	MSMEG5210	Exo-deoxyribonuclease VII, large subunit	2.737	0.0003	2
ML2088-a		MSMEG4807	Putative cytochrome P450	2.039	0.0004	7
ctpC-a		MSMEG5384	Cadmium-translocating P-type ATPase	1.789	0.0081	3
rpoT	*sigA*	MSMEG2759	RNA polymerase sigma factor, SigA	1.513	0.0000	2
rplE	*rplE*	MSMEG1464	Ribosomal protein L5	1.198	0.0002	2
ML2661-e	*fadD7*	MSMEG3702	Fatty acid coenzyme A ligase, FadD7	1.197	0.0051	1
fpg	*Fpg*	MSMEG2417	Formamidopyrimidine-DNA glycosylase	0.961	0.0078	7
ML1750		MSMEG2202	CHP	0.922	0.0098	10

[a] Gene identifier as defined in the MIAME compliance file (http://vbc.med.monash.edu.au/≈powell/M.smegmatis).
[b] TIGR annotation (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl).
[c] Function as per Tuberculist and Leproma (http://genolist.pasteur.fr). CHP, conserved hypothetical protein.
[d] Log_2 of the fold ratio.
[e] Functional classification as per Tuberculist and Leproma: 1, lipid metabolism; 2, information pathways; 3, cell wall and cell processes; 6, PE/PPE; 7, intermediary metabolism and respiration; 9, regulatory proteins; 10, conserved hypotheticals.

failure to recover a null *ctaD1* mutant was unlikely to be due to the essentiality of this gene but could be due to polar effects of the Δ*ctaDI*::*hyg* mutation on the downstream *serB2* gene, which may be essential (30).

We could only identify distinct *ctaD* alleles in the genome of *M. smegmatis* and not in any other sequenced mycobacterial species (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl; http://genolist.pasteur.fr). However, two or more *ctaD* alleles were also identified in the genomes of several other completely sequenced actinobacteria (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), including *Streptomyces*, and *Nocardia* species. *Paracoccus denitrificans* has similarly been found to contain two distinct *ctaD* genes (26, 38), suggesting that the presence of multiple *ctaD* genes is a reasonably common occurrence in bacterial genomes. In *P. denitrificans*, the *ctaDI* and *ctaDII* genes appear to be functionally interchangeable, implying the existence of CcO isoenzymes (26). Importantly, the microarray analysis suggested that *ctaDII* (MSMEG4435) is expressed in *M. smegmatis* under aerobic conditions. However, the extent (if any) of functional redundancy and/or interchangeability of the *M. smegmatis ctaD* alleles has yet to be determined.

The failure to recover allelic exchange mutants in the bc_1-aa_3 pathway of *M. tuberculosis* H37Rv suggests that the mutants were either severely attenuated or nonviable under the conditions tested. This conclusion is supported by the underrepresentation of transposon mutations in genes encoding the bc_1 complex (*qcrCAB*) and the CcO (*ctaC*, *ctaD*, *ctaE*) in high-density mutagenized libraries of *M. tuberculosis*, as determined by transcription site hybridization (30).

Up-regulation of *cyd* occurs in response to disruption of the bc_1-aa_3 respiratory pathway. In *M. smegmatis*, inactivation of the bc_1-aa_3 pathway at the level of the bc_1 complex or the CcO (data not shown) resulted in pronounced up-regulation of the *bd*-type terminal oxidase encoded by the *cydABDC* operon. Analogous observations have been made in *P. denitrificans*, where changes in electron distribution caused by blockage of a respiratory pathway affected the activities of terminal oxidase promoters (21). The viability of the *ctaC* and *qcrCAB* mutants of *M. smegmatis* attests to the adaptability of the respiratory network in this organism, which allows its energy requirements to be met by increased activity of the bioenergetically less efficient *bd*-type oxidase. In addition to *cydAB*, genes encoding a second putative *bd*-type oxidase have also been identified in *M. smegmatis* (MSMEG5584 and MSMEG5585) (16). The microarray analysis confirmed that MSMEG5584 is expressed in *M. smegmatis* grown aerobically, but unlike *cydA*, this gene was not differentially expressed in response to blockage of the bc_1-aa_3 pathway.

The apparent essentiality of the bc_1-aa_3 pathway in *M. tuberculosis* may be due to an inability of its aerobic respiratory network to adapt in a manner analogous to that of *M. smegmatis.* However, *cyd* gene expression in *M. tuberculosis* has been shown to be highly responsive to interference with the machinery that maintains the proton motive force, as evidenced by its upregulation by inhibitors of CcO and chemicals

that affect its maturation (5), by growth on palmitate (5), during adaptation to hypoxia (5, 40), and by protonophores (5). We therefore conclude that respiratory adaptation by *cyd* upregulation probably does occur in *M. tuberculosis* in response to disruption of the bc_1-aa_3 pathway, but the consequence of single-route electron flow to the bioenergetically less efficient cytochrome *bd*-type menaquinol oxidase (15) in this slow-growing mycobacterium may be such that mutant colonies might not be detectable under the conditions employed in this study (up to 54 days of incubation).

Other adaptations to rerouting of the electron flux through cytochrome *bd* oxidase. A broader view of the consequences of rerouting of the electron flux through the cytochrome *bd*-type menaquinol oxidase-terminating branch was obtained from comparative expression profiling of the bc_1 mutant and its parental wild type using a partial-genome microarray of *M. smegmatis* that is enriched for essential genes. In addition to *cydA*, two other genes known to be induced by hypoxia were also upregulated in the bc_1 mutant, *uspL* (MSMEG5230) and the homolog of Rv1592c (MSMEG3205). Interestingly, UspL is a member of the DosR regulon of *M. smegmatis* (20).

Although the partial-genome array also contained other genes identified by O'Toole et al. (20) as being hypoxically induced and regulated by DosR, including *hspX* (MSMEG3937), *uspM* (MSMEG3957), *dosS* (MSMEG5226), *dosR* (MSMEG5229) and *acg* (MSMEG5231), these genes were not found to be differentially expressed in the bc_1 mutant. Therefore, although the results of microarray analysis were suggestive of an overall increase in the reduced state of the respiratory chain, blockage of electron transport via the bc_1-aa_3 pathway by deletion of the bc_1 complex did not induce the DosR regulon of *M. smegmatis*. This observation suggests that the molecular signal(s) generated by deletion of the bc_1 complex is distinct from that generated by O_2 starvation, although some genes may respond to both, e.g., *cydA* (16) and *uspL* (this study). Current work is aimed at using the respiratory mutant strains as tools for investigating these molecular signals and their transduction pathways.

ACKNOWLEDGMENTS

This work was supported by grants from the Howard Hughes Medical Institute (International Research Scholar's grants to V.M. and R.L.C.), the Medical Research Council of South Africa, the National Research Foundation of South Africa, the World Health Organization TDR, the Australian National Health and Medical Research Council, the Australian Research Council, and the NIH (#AI43420 to H.R.).

Preliminary sequence data were obtained from the Institute for Genomic Research website at http://www.tigr.org. We thank Marty Voskuil and David Sherman for helpful discussions.

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