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The F_1F_o-ATP Synthase of *Mycobacterium smegmatis* Is Essential for Growth

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The F_1F_o-ATP synthase plays an important role in a number of vital cellular processes in plants, animals, and microorganisms. In this study, we constructed a Δ*atpD* mutant of *Mycobacterium smegmatis* and demonstrated that *atpD* encoding the β subunit of the F_1F_o-ATP synthase is an essential gene in *M. smegmatis* during growth on nonfermentable and fermentable carbon sources.

The F_1F_o-ATP synthases of bacteria play an important role in a number of vital cellular processes (3, 22). In aerobic bacteria, these enzymes are responsible for ATP generation via oxidative phosphorylation, leading to large amounts of ATP synthesized per substrate oxidized. In anaerobic bacteria, under nonrespiratory growth conditions, these enzymes work primarily as an ATPase, pumping protons to generate a proton motive force, and in some cases, this is coupled to pH homeostasis to prevent intracellular acidification (5, 10).

A recent study reported that the F_1F_o-ATP synthase of mycobacteria is the target of the first new antituberculosis drug family (diarylquinolines) to be discovered in 40 years (1). The authors suggest that the drug leads to ATP depletion and an imbalance in pH homeostasis in mycobacterial species, both contributing to decreased ability to survive. Other studies have implicated a potential role for the F_1F_o-ATP synthase in the physiology of mycobacteria at acidic pH (8, 17, 18). In this study, we sought to establish a role for this enzyme in the physiology of *Mycobacterium smegmatis* as a model for understanding its potential role in the biology of other mycobacterial species.

The *atpD* gene encoding the β subunit of the F_1F_o-ATP synthase is an essential gene in *M. smegmatis*. The putative *atp* operon coding for the F_1F_o-ATP synthase of *M. smegmatis* was identified in an unfinished genome database (www.tigr.org). The DNA sequence shows that this operon is similar to those of many bacteria and is colinear to the *atp* operon of *Mycobacterium tuberculosis* with the gene order *atpBEFHAGDC*. In order to disable the F_1F_o-ATP synthase of *M. smegmatis*, we chose to disrupt the *atpD* gene by allelic exchange mutagenesis by using a strategy adapted from Pelicic et al. (16).

M. smegmatis strain mc^2155 (23) and derived mutants (Table 1) were grown with agitation at 37°C in either Luria-Bertani medium supplemented with 0.05% (wt/vol) Tween 80 (Sigma Chemicals) (LBT) or Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) supplemented with sterile Middlebrook ADC enrichment (Becton Dickinson, Cockeysville, Md.). For solid medium, Middlebrook 7H11 was supplemented with ADC and glycerol (0.5% vol/vol) or LBT with 1.5% agar. Unless otherwise stated, *M. smegmatis* transformants were grown at 28°C for temperature-sensitive vector propagation and 40 to 42°C for allelic exchange mutagenesis. All subcloning steps were performed with *Escherichia coli* strain DH10B (Table 1) with culturing at 37°C in LB broth or 2× YT broth at an initial pH of 7.0 and LB agar (19). The *E. coli* plasmids used in this study are listed in Table 1.

To construct a vector to delete the *atpD* gene (i.e., pST100 Δ*atpD*::*aphA-3* [Fig. 1A]), primers were designed to amplify (Expand high-fidelity PCR system) approximately 1,000 bp flanking the putative *atpD* gene. The primer pair *AtpD4* (5′-AAATTTGAATTCCAAGAAGGCCTAGGTAACAG-3′) and *AtpD3* (5′-AAATTTGGTACCTACGCGACCCGCGGTCTTTT-3′) and the primer pair *AtpD2* (5′-AAATTTTCTAGACTGGACGACCTGGCGAAGAA-3′) and *AtpD1* (5′-AAATTTAAGCTTGTTGCCTGCGAAGTACCTCA-3′) were used to amplify the 5′ and 3′ flanks, respectively. The resulting amplicons were sequenced to confirm fidelity and were then ligated simultaneously with a kanamycin resistance cassette (KpnI-XbaI fragment containing *aphA-3* from pUC18K) (14) into EcoRI-HindIII-digested LITMUS 28 (New England Biolabs). The assembled *atpD* knockout construct (Δ*atpD*::*aphA-3* with 1,000 bp of homologous flanks) was then subcloned as a 2.8-kb AvrII fragment into the XbaI site of the delivery vector pPR23 (thus creating pST100) (Fig. 1A). The expected double crossover would result in a nonpolar deletion-insertion at the *atpD* locus, eliminating 94% of the *atpD* coding sequence in exchange for the kanamycin resistance marker. pST100 was electroporated into *M. smegmatis* mc^2155 (0.2-cm-gap cuvette at 2.5 kV, 1,000 Ω, and 25 μF) and transformants screened for gentamicin and kanamycin resistance and confirmed by electroduction (2).

Recombinants were selected by plating cells from a log-phase culture (optical density at 600 nm, 0.7 to 1.0) of a pST100 transformant (strain ST10) onto LBT agar supplemented with sucrose and kanamycin and incubated at 42°C. pST100 carries a temperature-sensitive mycobacterial origin of replication and *sacB* counterselection to facilitate screening for a double crossover (16). For mc^2155 derivatives, kanamycin, gentamicin, streptomycin, and spectinomycin were used at final

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or information[a]	Reference or source[b]
E. coli DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara-leu*)*7697 araD139 galU galK nupG rspL* λ$^-$	19
M. smegmatis		
mc^{2}155	Wild-type *ept-1*	23
ST10	mc^{2}155 carrying pST100 (Δ*atpD*::*aphA-3*), Km^r Gm^r	This study
ST24	mc^{2}155 Δ*atpD1*::*aphA-3*, Km^r	This study
ST34	ST24 harboring pST100, Km^r Gm^r	This study
ST35	ST34 with pST100 integrated (Δ*atpD2*::*pST100*), Km^r Gm^r	This study
ST36	ST35 harboring pST101, Km^r St^r/Sp^r	This study
ST40	mc^{2}155 harboring pST220 (*atpB-lacZ*), Km^r	This study
Plasmids		
pUC18K	Contains *aphA-3* cassette, Km^r	14
LITMUS 28	*E. coli* cloning vector, Ap^r	NEB
pPR23	*E. coli*-mycobacterial shuttle vector, *oriM* temp^s, *sacB* Gm^r	16
pCG76	Shuttle vector, temp^s, St^r/Sp^r	27
pMV261	Shuttle vector, BCG Hsp60 promoter, Km^r	25
pJEM15	Shuttle vector, Km^r	26
pLatpD	LITMUS 28 harboring Δ*atpD1*::*aphA-3*, Ap^r	This study
pST100	pPR23 harboring Δ*atpD1*::*aphA-3*, Km^r Gm^r	This study
pST101	pCG76 harboring P_{Hsp60}-*atpD*$^+$ fusion, St^r/Sp^r, temp^s	This study
pST200	pMV261 harboring *atpD*$^+$, Km^r	This study
pST220	pJEM15 harboring *atpB-lacZ* fusion, Km^r	This study

[a] Km^r, kanamycin resistance; Gm^r, gentamicin resistance; St^r, streptomycin resistance; Sp^r, spectinomycin resistance; Ap^r, ampicillin resistance; temp^s, temperature sensitivity.
[b] NEB, New England Biolabs.

concentrations of 20, 5, 20, and 20 μg/ml, respectively. Sucrose was used at a final concentration of 10% (wt/vol). Colonies that appeared were screened for gentamicin sensitivity (loss of vector) and kanamycin resistance (allelic exchange of *atpD* for Δ*atpD*::*aphA-3*). Colonies displaying the desired phenotype (kanamycin resistance, gentamicin resistance, and sucrose resistance) were analyzed by colony blot hybridization to confirm the absence of the delivery vector (pPR23), indicating that the putative mutants had likely undergone double crossover. Genomic DNA from putative Δ*atpD* mutants displaying the desired phenotype (kanamycin resistance, gentamicin resistance, sucrose resistance, and growth at 42°C) was digested with SmaI, and the fragments were separated by agarose gel electrophoresis, blotted onto nylon membrane and then probed with the 3′ flanking fragment, designated RF (see Fig. 1B). To further confirm the loss of the wild-type *atpD* allele, primer pair *AtpD4* and *AtpD1* or *AtpDcompF* and *AtpDcompR* was used to amplify the region containing the *atpD* loci, with the product size reflecting the presence of wild-type *atpD* or the deletion of *atpD*.

Southern hybridization analysis and PCR revealed that the putative mutants carried a second *atpD* locus (designated *atpD2*). SmaI-digested chromosomal DNA, probed with RF (Fig. 1B), hybridized to a single SmaI fragment of approximately 10.7 kb in the parental strain (Fig. 2, lane 2). In these mutants, we expected a double crossover with pST100 to generate a single 2.7-kb hybridizing band. Unexpectedly, two hybridizing bands were found in all mutants screened: the expected 2.7-kb band and an additional 10.7-kb band (Fig. 2, lane 3). These results suggested that *atpD* and the surrounding region have been duplicated in *M. smegmatis*, and only one copy of *atpD* had been successfully deleted from this mutant (designated ST24 [Δ*atpD1*::*aphA-3*]). It has been reported that *M. smegmatis* contains a large duplication (approximately 250 kb or greater) in its genome (7), and this may explain the two copies of *atpD* observed here. The growth rate and cell yield of ST24 were identical to those of strain mc^{2}155 in M63 minimal medium containing either succinate or glucose, suggesting that a second copy of *atpD* (designated *atpD2*) was indeed present (data not shown).

Upon discovery of *atpD2*, pST100 was reintroduced into ST24 (creating ST34) and double crossover at the *atpD2* locus was executed through a two-step approach (Fig. 1B): (i) pST100 was forced to integrate into *atpD2* by selection for gentamicin resistance at 42°C (generating strain ST35), and (ii) *atpD* was then supplied in *trans* on the Δ*atpD* complementing vector pST101, allowing the resolution of the integrated pST100 backbone and simultaneous allelic exchange at *atpD2* (generating strain ST36).

Putative *atpD* double knockouts were screened for kanamycin and sucrose resistance and then confirmed by Southern blot analysis and PCR. Integration of pST100 at *atpD2* resulted in a shift from a 10.7-kb hybridizing band to a 16-kb hybridizing band (Fig. 2, lane 4). The hybridization profile also confirmed that only two copies, rather than multiple copies, of *atpD* are present in the genome of *M. smegmatis*. Integrants exhibited slow growth rates and poor cell yields, implying that the integration was polar. In support of this, Southern analysis showed that all legitimate integrants had the same genotype, indicating a bias toward the continued expression of *atpD* from an intact *atp* operon and promoter (data not shown). To select for a second crossover (and resolution of the intervening sequence),

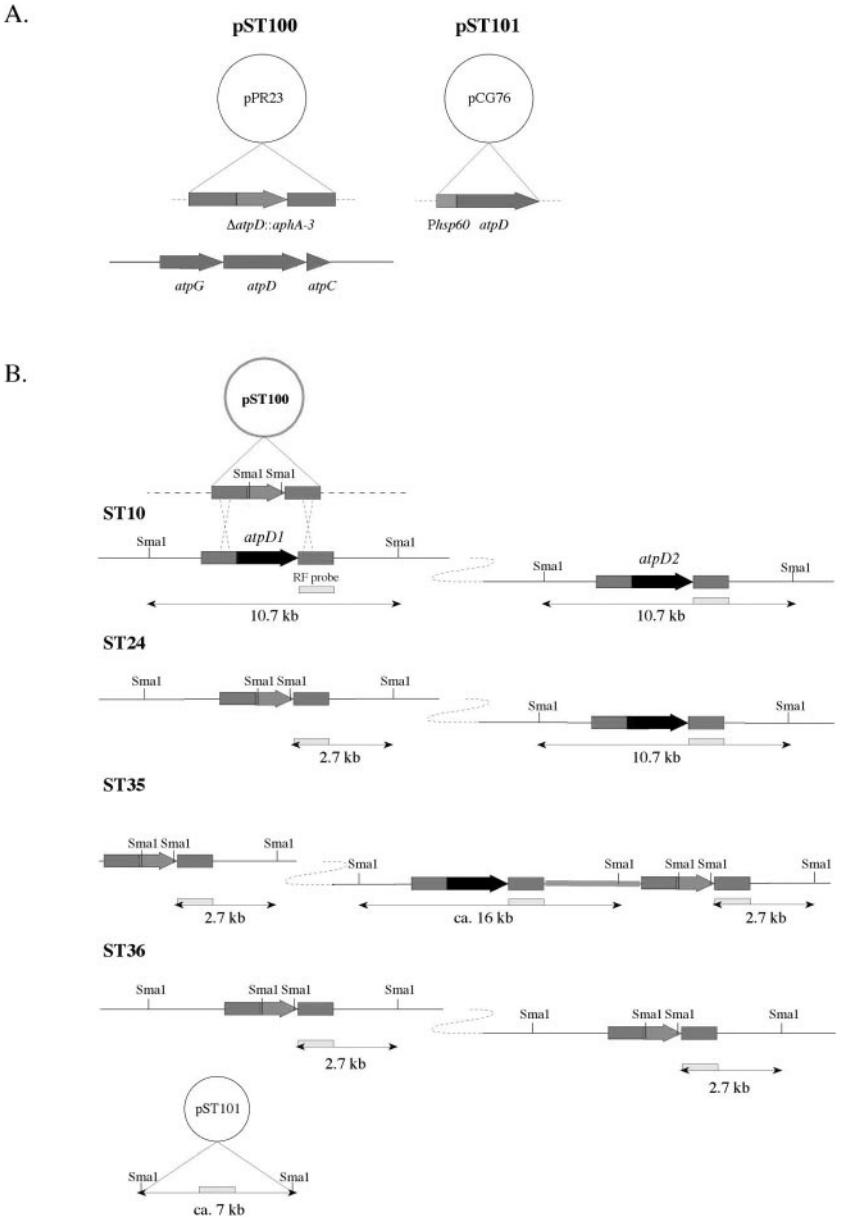

FIG. 1. (A) Key plasmids used in the generation of ST36 (Δ*atpD*::*aphA-3*). Allelic exchange delivery vector pST100 carries DNA amplified from the *atp* operon of *M. smegmatis* mc^{2}155. The in *trans* Δ*atpD* complementing vector pST101 carries *atpD* under the constitutive control of the *Mycobacterium bovis* BCG Hsp60 promoter (P*hsp60*). (B) The genetic consequences of homologous recombination with Δ*atpD*::*aphA-3* (see the text for detailed explanation).

ST35 was grown on kanamycin and sucrose at 42°C. This consistently resulted in colonies with gentamicin resistance (336/336 tested), indicating that the screen was ineffective (likely due to *sacB* mutation) or that *atpD* was essential for growth of *M. smegmatis*. To address the latter hypothesis, an *atpD* complementing vector (temperature-sensitive origin of replication), pCG76 harboring *atpD*$^+$ (pST101), was constructed to increase the probability of a double crossover at *atpD2*. The Δ*atpD* complementing plasmid, pST101 (Fig. 1A), was constructed by amplifying *atpD* with primers *AtpDcompF* (5′-AAATTTAGATCTGCAAGCCCCTTTCAGGAAGC-3′) and *AtpDcompR* (5′-AAATTTAAGCTTGACATGGCAATCAC AGCTTG-3′) and cloning the resulting product as a BglII-HindIII fragment immediately downstream of the BCG Hsp60 promoter (P_{Hsp60}) carried by pMV261 (25), thus allowing the constitutive expression of *atpD*. The P_{Hsp60}-*atpD* fusion (P_{Hsp60}-*atpD*) was then ligated (as a XbaI-NheI fragment) into the XbaI site of pCG76.

Introduction of pST101 (*atpD* in *trans*) into ST35 resulted in the spontaneous excision of the integrated pST100 plasmid backbone, the hybridization profiles indicating that pST101 transformants had undergone a double crossover at *atpD2* (Fig. 2, lane 5; see Fig. 1B for schematic representation). This is consistent with the incompatibility of pAL5000-derived vec-

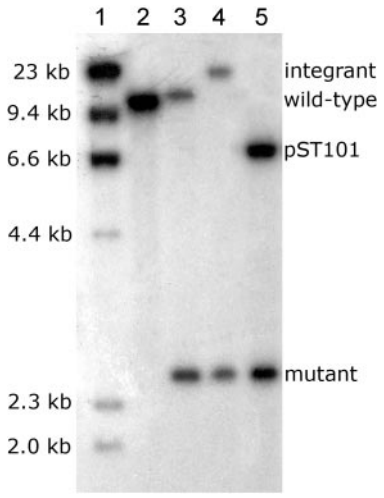

FIG. 2. Southern hybridization of SmaI-digested genomic DNA was used to confirm double crossover events, which generate a 2.7-kb fragment instead of the wild-type 10.7-kb fragment. In ST35 (Δ*atpD1*; *atpD2*::pST100), a 16-kb band is generated by the integration of pST100 in place of the wild-type fragment. The double knockout mutant ST36 (Δ*atpD1* Δ*atpD2* pST101) carries *atpD* in *trans*, thus shows the 2.7-kb double crossover band (a doublet) and a 7.0-kb band derived from pST101. Lane 1, λ DNA (HindIII digest); lane 2, mc^{2}155 (wild type); lane 3, ST24; lane 4, ST35; lane 5, ST36.

tors described by Stolt and Stoker (24). A double crossover mutant was selected and designated ST36 (Fig. 2, lane 5).

Characterization of the Δ*atpD*::*aphA-3* mutant. To determine if *atpD* was essential in *M. smegmatis*, ST36 cultures were grown at 28°C and 42°C in LBT supplemented with 20 mM glucose (fermentable carbon source) and compared to the single-Δ*atpD1*::*kan* mutant ST24 and the wild-type strain mc^{2}155 (Fig. 3A and B). A starter culture of ST36 was grown to mid-log phase (optical density at 600 nm, <1.0) at 28°C in LBT and then was used to inoculate 5-ml LBT broths (supplemented with 20 mM glucose) prewarmed to either 28°C or 42°C. Growth was followed by measuring the optical density at 600 nm. The ST24 Δ*atpD1* mutant and wild-type strain mc^{2}155 were used as controls in all experiments.

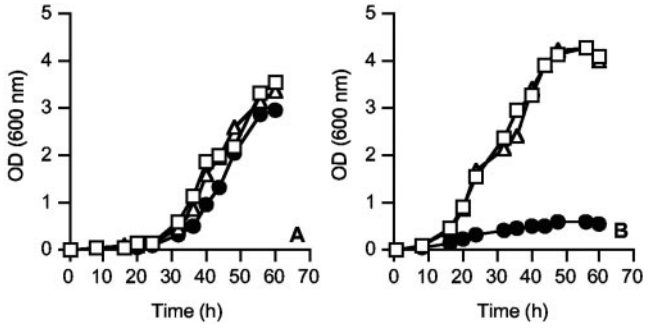

FIG. 3. The effect of conditional (temperature-sensitive) *atpD* expression on the growth of ST36. The growth of ST36 (●) at 28°C (A) and 42°C (B). Wild-type mc^{2}155 (□) and ST24 (Δ) were used as control organisms. All cultures were grown in LBT containing 20 mM glucose. The growth curves shown are representative of three individual experiments in which the data did not differ by more than 10%. OD, optical density.

The growth of the single and double *atpD* knockout mutants at 28°C proceeded at the same rate as that of the wild-type strain mc^{2}155 (Fig. 3A). The growth rates of ST24 and the wild-type mc^{2}155 were also the same at 42°C. ST36 did not show significant growth at 42°C (pST101 supplying *atpD* in *trans* is temperature sensitive at 42°C and cannot replicate), thus demonstrating that *atpD* was indeed essential for growth (Fig. 3B). Equivalent results were obtained with succinate as the sole carbon and energy source (data not shown).

Expression of *atpB-lacZ* is constitutive and not regulated by growth rate or low pH. To examine the role of F_1F_o-ATPase in the physiology of *M. smegmatis*, we studied the expression of the *atp* operon under various growth conditions. To monitor F_1F_o-ATPase expression, we constructed an in *trans* transcriptional *atpB-lacZ* fusion (strain ST40). To construct an *atpB-lacZ* fusion, approximately 900 bp upstream of the putative *atpB* gene was amplified by using the primer pair *PatpOF* (5′-AATGTCGGATCCGCAGAAAGTCGTCAGGTCAG-3′) and *PatpOR* (5′-ACGGCAGGATCCAGAATGGTGTC GCCATTGAA-3′). The resulting amplicon was ligated as a BamHI fragment into the promoterless *lacZ* vector pJEM15 (26) to create an *atpB-lacZ* transcriptional fusion (plasmid pST220). The construct was electroporated into *M. smegmatis* mc^{2}155 and putative transformants (designated ST40) confirmed by PCR and DNA sequencing.

The transcriptional activity of *atpB-lacZ* was then evaluated under various growth regimens. For *atpB-lacZ* expression studies, *M. smegmatis* was cultured in M63 minimal medium supplemented with 0.05% (wt/vol) Tween 80 and a 40 mM concentration of glucose, glycerol, succinate, glutamate, or fructose. Cells were harvested and *atpB-lacZ* expression determined by β-galactosidase assays as previously described (26). Data given in Miller units (MU) are the mean values of three independent experiments with the standard deviations of the means reported. When strain ST40 was grown over the pH range of 5.5 to 8.0, no significant change in *atpB-lacZ* activity was observed (approximately 250 ± 35 MU). Furthermore, if cells were resuspended at a very acidic pH (pH 3.0 for 2 h), no upregulation of *atpB-lacZ* expression was observed (data not shown).

To study the effect of the carbon source on *atpB-lacZ* expression, ST40 was grown in M63 medium with various carbon sources (the specific rates of growth on each carbon source are as follows: succinate, 0.17 h^{-1}; glucose, 0.12 h^{-1}; fructose, 0.21 h^{-1}; glutamate, 0.09 h^{-1}; and glycerol, 0.27 h^{-1}). The level of *atpB-lacZ* expression on nonfermentable carbon sources (i.e., succinate, 361 ± 58 MU; glutamate, 358 ± 32 MU; and glycerol, 342 ± 49 MU) was high compared to those of the substrates that were not strictly coupled to oxidative phosphorylation (i.e., the levels of expression for glucose and fructose were 274 ± 25 MU and 216 ± 30 MU, respectively). No discernible trend between growth rate and *atpB-lacZ* expression was observed.

These results demonstrate that *atpD* and, therefore, the F_1F_o-ATP synthase are essential for growth of *M. smegmatis* on both fermentable and nonfermentable carbon sources. This finding is congruent with those of Sassetti et al. (21), in whose study *atpD* (and the other genes in the *atp* operon) were reported to be essential genes by Himar1-based transposon mutagenesis in *M. tuberculosis* H37Rv (21). The essentiality of the

F_1F_o-ATP synthase in the growth of species of other bacterial genera (e.g., *Helicobacter pylori*, *Lactococcus lactis*, and *Listeria monocytogenes*) has also been documented (4, 11, 13); however, in none of these studies has the essential requirement been defined experimentally.

In other bacteria, the F_1F_o-ATP synthase has been shown to be dispensable for growth on fermentable carbon sources (6, 9, 20, 29), in which increased glycolytic flux can compensate for the loss of oxidative phosphorylation. This strategy does not appear to be exploited by *M. smegmatis*: the F_1F_o-ATP synthase is essential for growth even on fermentable substrates, suggesting that ATP production from substrate level phosphorylation alone, despite increased glycolytic flux, may be insufficient to sustain the growth of these bacteria. This would be reflected by an extraordinarily high value for the amount of ATP required to synthesize a mycobacterial cell, a possibility that requires further investigation. Alternatively, or in conjunction with a high ATP demand for growth, the ATP synthase may be an obligatory requirement for the oxidation of NADH by providing a sink for translocated protons during NADH oxidation coupled with oxygen reduction. Such strict coupling would imply that mycobacteria do not support uncoupled respiration: either they lack a conduit for proton reentry in the absence of the F_1F_o-ATP synthase or they are unable to adjust the proton permeability of the cytoplasmic membrane to allow a futile cycle of protons to operate. Recently, we have shown that the cytoplasmic membrane of *M. smegmatis* is extremely impermeable to protons (27).

The oxidation of NADH by aerobic bacteria is critical for continuous metabolic flux, and in the absence of fermentative metabolism, NADH oxidation will be carried out primarily by NADH dehydrogenases operating in conjunction with a respiratory chain. Weinstein et al. (30) have identified genes for two classes of NADH:menaquinone oxidoreductases in the genome of *M. tuberculosis*. NDH-1 is a proton-translocating NADH dehydrogenase (encoded by *nuoABCDEFGHIJKLMN*) and NDH-2 is a non-proton-translocating NADH dehydrogenase present in two copies (*ndh* and *ndhA*) (30). Mutagenesis studies have established that both NDH-1 (21) and *ndhA* (12) are dispensable for growth in vitro, but the lack of a viable strain with a disrupted or deleted *ndh* gene suggests that this gene is essential for growth. Deleterious point mutations in *ndh* of *M. smegmatis* are pleiotropic, conferring temperature-sensitive growth arrest and multiple-amino-acid auxotrophy. Some mutants also have a 25-fold-reduced NADH dehydrogenase activity, implying that NDH-2 is the primary enzyme responsible for NADH oxidation and it is essential for the viability of *M. smegmatis* (15, 28).

Several new antitubercular drugs have recently been reported (1, 30). It is particularly noteworthy that both classes of drugs target oxidative phosphorylation in mycobacteria. The first class, diarylquinolines (i.e., R207910) target the F_1F_o-ATP synthase (1), and the second class, phenothiazine analogs, target the NADH:menaquinone oxidoreductase (30), suggesting that NADH oxidation via aerobic respiration coupled with oxidative phosphorylation is essential for the growth of mycobacteria. Future research will focus on the relationship between these two important metabolic processes in mycobacteria.

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