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Roles of the two type II NADH dehydrogenases in the survival of *Mycobacterium tuberculosis* in vitro

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ABSTRACT

Most bacteria are able to generate sufficient amounts of ATP from substrate level phosphorylation, thus rendering the respiratory oxidative phosphorylation non-critical. In mycobacteria, including *Mycobacterium tuberculosis*, ATP generation by oxidative phosphorylation is an essential process. Of the two types of NADH dehydrogenases (type I and type II), the type II NADH dehydrogenase (Ndh) which is inhibited by phenothiazines has been thought to be essential. In *M. tuberculosis* there are two Ndh isozymes (Ndh and NdhA) coded by *ndh* and *ndhA* genes respectively. Ndh and NdhA share a high degree of amino acid similarity. Both the enzymes have been shown to be enzymatically active and are inhibited by phenothiazines, suggesting a functional similarity between the two. We attempted gene knockout of *ndh* and *ndhA* genes in wild type and merodiploid backgrounds. It was found that *ndh* gene cannot be inactivated in a wild type background, though it was possible to do so when an additional copy of *ndh* was provided. This showed that in spite of its apparent functional equivalence, NdhA cannot complement the loss of Ndh in *M. tuberculosis*. We also showed that NdhA is not essential in *M. tuberculosis* as the *ndhA* gene could be deleted in a wild type strain of *M. tuberculosis* without causing any adverse effects in vitro. RT-PCR analysis of in vitro grown *M. tuberculosis* showed that *ndhA* gene is actively transcribed. This study suggests that despite being biochemically similar, Ndh and NdhA play different roles in the physiology of *M. tuberculosis*.

1. Introduction

Tuberculosis (TB) is a major cause of morbidity and mortality in many parts of the world especially in underdeveloped and developing countries. TB, caused by *Mycobacterium tuberculosis* (Mtb), is a complex disease and requires treatment with a combination of four drugs for six months. The long duration of treatment is probably a reflection of multiple physiological states of bacteria present in a variety of lesions in TB patients (Barry et al., 2009). It has been observed that slow replicating or non-replicating Mtb bacteria are refractory to the action of anti-TB drugs (Xie et al., 2005). The long duration of treatment often leads to non-compliance on the parts of the patients, which exposes bacteria to sub-inhibitory concentrations of the drugs. These conditions can favor the emergence of drug resistant forms of bacteria. Recent epidemiological data indicates that the incidence of drug resistant Mtb has been increasing all over the world (Mesfin et al., 2014; Wells et al., 2007). Some strains of Mtb are resistant to more than two (MDR) or almost all the first line and second line anti-TB drugs (XDR) (Abubakar et al., 2013). This calls for urgent efforts to discover new drugs for treating drug resistant forms of Mtb. For compounds to be active on drug resistant Mtb, it is imperative that they have a novel mechanism of action (MoA). In order to achieve this objective, new targets and pathways need to be analyzed and validated for developing anti-TB inhibitors. For reducing the duration of therapy it is important that the drugs should also kill slowly replicating or non-replicating forms of Mtb (Fattorini et al., 2013; Hurdle et al., 2011). Hence, physiological processes which are active during dormant or non-replicating phase could be targeted for identifying Mtb inhibitors.

Oxidative phosphorylation is a universal process of energy generation in living cells of eukaryotes and prokaryotes. Bacteria have two major modes of energy generation: 1. Substrate level phosphorylation as seen in TCA cycle and 2. Oxidative phosphorylation (Cook et al., 2009). Most bacteria are able to generate sufficient amounts of ATP from substrate level phosphorylation which makes the respiratory oxidative phosphorylation pathway non-critical (Santana et al., 1994). For this reason the oxidative phosphorylation pathway is not essential for the survival of most bacterial species. Hence, oxidative phosphorylation

Abbreviations: TB, tuberculosis; Mtb, *Mycobacterium tuberculosis*; MDR/XDR, multiple/extremely drug resistant Mtb; NDH, NADH dehydrogenase; MoA, mechanism of action; INH, isoniazid; KO, knockout; SCO, single cross-over; DCO, double cross-over; WT, wild type; RT-PCR, reverse transcriptase PCR; Mero, merodiploid.

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is generally not considered as a valid target for discovering antibacterials. However, the recently FDA approved anti-TB drug TMC-207 (Bedaquiline) works by inhibiting ATP synthase (Andries et al., 2005; Segala et al., 2012). This suggests that mycobacteria differ from other bacteria in the process of generating energy in the form of ATP. In other words, in mycobacteria, including *M. tuberculosis*, ATP generation by oxidative phosphorylation is an essential process. In fact, *atpD* gene coding for β subunit of ATP synthase has been shown to be essential in *Mycobacterium smegmatis* (Tran and Cook, 2005). Among various steps in oxidative phosphorylation, type I and type II NADH dehydrogenases play the role of oxidation of NADH to generate NAD (Bald and Koul, 2010). As is the case with ATP synthase, type II Ndh is also a non-essential enzyme in most bacteria (Bald and Koul, 2010). However, type II Ndh inhibitors with cellular activity against human parasites *Toxoplasma gondii* and *Plasmodium falciparum* have been reported recently (Saleh et al., 2007), which proves that type II Ndh is an essential enzyme in these parasites. In a recent study it was shown that type I dehydrogenases, though not essential for survival in vitro, are required for intracellular survival and virulence of Mtb (Miller et al., 2010). In Mtb two isozymes of type II NADH dehydrogenases (Ndh), Ndh and NdhA, have been reported. The recombinant forms of both the enzymes have been purified from *Escherichia coli* and *M. smegmatis* and have been characterized biochemically (Teh et al., 2007; Weinstein et al., 2005). Chlorpromazine, a phenothiazine used for treating *Mycobacterium leprae* infections, has been shown to inhibit the biochemical activity of Ndh and NdhA (Weinstein et al., 2005). Enzyme inhibitors of bacterial Ndh have recently been reported (Mogi et al., 2009; Shirude et al., 2012). However, the essentiality of *ndh* genes in Mtb has not been proven unequivocally. Interestingly, some rare mutations causing isoniazid (INH) resistance in clinical strains of Mtb have been mapped to Ndh (Boonaiam et al., 2010; Cardoso et al., 2007; Hazbón et al., 2006; Lee et al., 2001). It has been postulated that any change in NAD/NADH ratio inside the cell leads to the loss of viability (Miesel et al., 1998; Vilchèze et al., 2005), which suggests that NADH dehydrogenases play a crucial role in the mycobacterial physiology by maintaining this ratio. Are both Ndh and NdhA equally important for maintaining NAD/NADH balance in Mtb? In order to find an answer to this, we have performed targeted gene knockouts (KOs) of *ndh* and *ndhA* genes in Mtb. Surprisingly, *ndh* could not be inactivated unless an additional copy of the gene was present in the cell. On the other hand *ndhA* could be inactivated in the wild type background and thus was proven to be non-essential. This suggests that the physiological roles of the two seemingly similar NADH dehydrogenases in Mtb are not identical. We propose that Ndh should be primarily targeted for obtaining inhibitors.

2. Materials and Methods

2.1. Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

Table 1
Strains and plasmids used in this study.

Strain/plasmid	Genotype/relevant features	Source
E. coli DH5α	*endA1*, *hsdR17*, *supE44*, *recA1*, *relA1*, (*lacZYA-argF*)	Lab stock
M. tuberculosis H37Rv (ATCC 27294)	Virulent strain of *M. tuberculosis*	Lab stock
pAZI0290	*E. coli* ori, *sacB*, Hyg^r	Awasthy et al. (2010)
pAZI9018b	Replicating mycobacterial expression vector with *pTrc* promoter	Kaur et al. (2009)
pAZI0333	Integrating mycobacterial expression vector with *pTrc* promoter	This study
pAZI0313	1.6 Kb DNA fragment with Δ*ndh* flanked by upstream and downstream sequences cloned into SspI–ScaI sites of pAZI0290	This study
pBAN0433	1.7 Kb DNA fragment with Δ*ndhA* flanked by upstream and downstream sequences cloned into SspI–ScaI sites of pAZI0290	This study
pBAN0512	*ndh* gene of *M. tuberculosis* cloned into NheI–PacI sites of pAZI0333	This study

2.2. Mtb Growth Conditions

The Mtb cultures were routinely grown in 7H9 medium (Difco) containing albumin dextrose complex (ADC). Hygromycin (50 μg/ml) or kanamycin (20 μg/ml) was added to the culture medium when required.

2.3. Gene Knockout (KO) Plasmid Constructs

The making of *ndh* and *ndhA* KO constructs was outsourced to Syngene International (Bangalore, India). The construct used for KO of *ndh* gene consisted of 500 bp of region upstream of *ndh*, a deletion of 298 bp in *ndh* (starting from 302nd base pair of the gene) followed by 800 bp of remaining part of *ndh*. The final construct was cloned into SspI–ScaI sites of pAZI0290 to obtain pAZI0313. For generating a complementation construct, full length *ndh* was cloned behind *pTrc* promoter of pAZI0333 using NheI–PacI sites to obtain pBAN0512. pAZI0333 was constructed by replacing the hsp promoter of pAZI272 (Awasthy et al., 2010) with the *pTrc* promoter region of pAZI9018b (Kaur et al., 2009). The sequences of the cloned DNA fragments were confirmed by sequencing (Microsynth). Similarly the *ndhA* KO construct consisted of a 596 bp of region upstream of *ndhA*, a deletion of 301 bp in *ndhA* (starting from 202nd base of the gene) followed by a stop codon and 800 bp of *ndhA*. The final construct was cloned into SspI–ScaI sites of pAZI0290 to obtain pBAN0433.

2.4. Gene KO of ndh/ndhA

A two-step method involving formation of a single cross over (SCO) followed by a double cross over (DCO) recombination described earlier (Awasthy et al., 2010; Parish and Stoker, 2000) was followed for inactivating *ndh* and *ndhA* genes of Mtb. Briefly, the KO plasmid construct was electroporated into Mtb and the colonies growing in the presence of hygromycin were analyzed by PCR. The SCO recombinants confirmed by PCR were allowed to undergo DCO recombination by growing the cultures in the absence of hygromycin for 4 weeks and plating them in the presence of 2% sucrose. The putative DCO recombinant colonies growing in sucrose containing plates were screened for the presence of deleted copy of *ndh* or *ndhA* gene in the chromosome and the loss of the plasmid by PCR. For constructing a *ndh* merodiploid strain, pBAN0512 carrying *ndh* gene was electroporated into the SCO strain and colonies were selected in the presence of hygromycin and kanamycin. The merodiploid strain was allowed to undergo DCO recombination as described above for the SCO strain. The gene KOs were confirmed by a combination of PCRs.

2.5. DNA Amplification by PCR

The SCO and DCO recombinants of Mtb *ndh*/*ndhA* gene were screened by colony PCR as described earlier (Awasthy et al., 2010) using Taq DNA polymerase (Genei, India). Denaturation and extension reactions were carried out at 94 °C and 72 °C respectively. The annealing temperature and extension time for each PCR were decided by considering the Tm of the primer pair and expected length of the PCR product respectively. The sequences of primers used for PCR amplifications are given in Table S1.