Biosynthesis and Recycling of Nicotinamide Cofactors in *Mycobacterium tuberculosis*  
AN ESSENTIAL ROLE FOR NAD IN NONREPLICATING BACILLI*†"  
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Despite the presence of genes that apparently encode NAD salvage-specific enzymes in its genome, *Mycobacterium tuberculosis* has been previously thought that *Mycobacterium tuberculosis* can only synthesize NAD *de novo*. Transcriptional analysis of the *de novo* synthesis and putative salvage pathway genes revealed an up-regulation of the salvage pathway genes *in vivo* and *in vitro* under conditions of hypoxia. [14C]Nicotinamide incorporation assays in *M. tuberculosis* isolated directly from the lungs of infected mice or from infected macrophages revealed that incorporation of exogenous nicotinamide was very efficient in *in vivo*-adapted cells, in contrast to cells grown aerobically *in vitro*. Two putative nicotinic acid phosphoribosyltransferases, PncB1 (Rv1330c) and PncB2 (Rv0573c), were examined by a combination of *in vitro* enzymatic activity assays and allelic exchange studies. These studies revealed that both play a role in cofactor salvage. Mutants in the *de novo* pathway died upon removal of exogenous nicotinamide during active replication *in vitro*. Cell death is induced by both cofactor starvation and disruption of cellular redox homeostasis as electron transport is impaired by limiting NAD. Inhibitors of NAD synthetase, an essential enzyme common to both recycling and *de novo* synthesis pathways, displayed the same bactericidal effect as sudden NAD starvation of the *de novo* pathway mutant in both actively growing and nonreplicating *M. tuberculosis*. These studies demonstrate the plasticity of the organism in maintaining NAD levels and establish that the two enzymes of the universal pathway are attractive chemotherapeutic targets for active as well as latent tuberculosis.

Tuberculosis remains the leading killer in the world because of a single infectious pathogen. With a third of the world population estimated to be latently infected with *Mycobacterium tuberculosis*, new drugs are urgently required to shorten the duration of therapy, eradicate multiple drug-resistant strains, and target latent, nonreplicating bacilli (1). Current therapeutic regimens require 6–9 months of chemotherapy and target aspects of cell wall biosynthesis, translation, transcription, or DNA topology. Current antitubercular drugs have diminished or even minimal effect against nonreplicating bacilli (2). This diminished effect may reflect the decreased activity of the various target enzymes under *in vivo* or nonreplicating conditions. Although some metabolic pathways are presumed to be important for maintenance of viability under all conditions, even when the bacilli are nonreplicating, little is known about adaptation of *M. tuberculosis* metabolism to *in vivo* conditions (3).

Cofactor biosynthesis is a rich source of potential drug targets because of the essential nature of these coenzymes throughout metabolism (4). NAD is an essential cofactor that is required for redox balance (5) and energy metabolism, as well as for the activity of the NAD-dependent DNA ligase in prokaryotes (6), protein ADP-ribosylases (7, 8), protein deacetylation (9), and as a substrate in cobalamin biosynthesis (10) and for calcium homeostasis (11). NAD can be synthesized *de novo* in prokaryotes from aspartate and dihydroxyacetone phosphate in an oxygen-dependent pathway or it can be scavenged or recycled by a variety of pathways (12, 13). For pathogens, this recycling pathway offers the possibility of obtaining this cofactor directly from their host. Recently it was also discovered that some prokaryotes synthesized NAD *de novo* from tryptophan (14), a pathway that had previously been considered unique to eukaryotes. The Preiss-Handler pathway (15) is a recycling pathway that occurs in many microorganisms and consists of nicotinate phosphoribosyltransferase (EC 2.4.2.11 or PncB) as well as the two enzymes of the universal pathway, nicotinic acid mononucleotide adenyltransferase (EC 2.7.7.18 or NaMNAT, encoded by *nadD*) and NAD synthetase (EC 6.3.5.1, encoded by *nadE*) (Fig. 1A). Some microbes depend on nicotinic acid for NAD synthesis because of the absence of some or all of the *de novo* pathway genes (16). Nicotinic acid is formed by the activity of nicotinamidase (EC 3.5.1.19 or PncA) on nicotinamide.
Nicotinamide and nicotinic acid can be scavenged from the environment but are also generated through the intracellular breakdown of NAD. NAD can be degraded by a variety of enzymes, including NAD glycohydrolase, DNA ligase, NAD pyrophosphatase, NAD(P)⁺ nucleosidase, poly(ADP-ribose) polymerase, mono-ADP-ribosyltransferase, and NAD pyrophosphatase (13). In an alternative nondeamidating salvage pathway, nicotinamide phosphoribosyltransferase (encoded by *nadV*) salvages nicotinamide directly with the resulting NMN subsequently converted to NAD by the MNM adenyltransferase activity of NadR (17). A third recycling pathway includes the conversion of exogenously scavenged pyridine nucleotides to NAD (17, 18).

In *M. tuberculosis*, the enzymatic machinery of the NAD de novo biosynthetic pathway has been identified (19) and is likely to be essential in vitro based on Himar–transposon mutagenesis studies (20). *M. tuberculosis* is biochemically identified, in part, by a characteristic accumulation of nicotinic acid (21–23) and by the presence of a nicotinamidase (encoded by *pncA*) that has been implicated in the hydrolysis of the nicotinamide analog pyrazinamide, an important component of front-line *M. tuberculosis* chemotherapy (24). Thus far there is no direct evidence that pyrazinonic acid acts as a metabolic poison of any aspect of NAD metabolism (25). However, despite the expression of this potent nicotinamidase, previous studies have indicated that the Preiss–Handler recycling pathway was not functional in this organism based on the apparent lack of nicotinate incorporation into NAD (13, 26). In addition, *pncA* can readily be inactivated in clinical strains that acquire pyrazinamide resistance without the apparent loss of fitness (27). NAD glycohydrolase activity has also been reported in *M. tuberculosis* cultures (26, 28), but the corresponding gene has not yet been identified.

The NAD biosynthetic pathway is thought to be an ideal drug target (4) with the steps shared by the de novo and recycling pathway posing candidate enzymes for therapeutic intervention. NAD, like most other phosphorylated compounds, cannot be transported across most bacterial cell envelopes, although there are notable exceptions (18, 29). However, in most bacteria, NAD is synthesized either de novo or is salvaged through the Preiss–Handler pathway. In this study we sought to determine the relative importance of de novo synthesis and nicotinamide scavenging from the host in *M. tuberculosis* under conditions similar to those likely to be encountered by the bacterium during disease in humans. Overall, the data show clearly that recycling of exogenously acquired nicotinamide is an important and functional pathway in *M. tuberculosis*; however, the organism shows considerable flexibility in switching between recycling and de novo synthesis of NAD suggesting that interrupting either one alone would be nonlethal. Therefore, only the two common enzymes shared by both pathways (NadD and NadE) are viable drug targets.

**EXPERIMENTAL PROCEDURES**

**Growth of Strains—Escherichia coli** strains were grown in Luria broth. Cloning and plasmid preparation were performed in *E. coli* DH5α, whereas proteins were expressed in *E. coli* BL21(DE3)pLysS cells. *M. tuberculosis* strains were cultured in Middlebrook 7H9 broth, which consisted of Middlebrook 7H9 broth base/albumin/dextrose/NaCl (ADC) enrichment, 0.2% glycerol, 0.05% Tween 80. Middlebrook 7H11 agar consisted of Middlebrook 7H11 medium supplemented with oleic acid/ADC (OADC) enrichment and 0.4% glycerol. Antibiotics were used at the following concentrations (*Mycobacterium/E. coli*): hygromycin 50 μg/ml/200 μg/ml, kanamycin 25 μg/ml/50 μg/ml, and gentamycin 10 μg/ml/10 μg/ml. Anaerobic and microaerophilic cultures of *M. tuberculosis* were set up as described by Wayne (30) in Dubos medium, which consisted of Dubos broth base supplemented with Dubos ADC enrichment and 0.05% Tween 80.

**Synthesis of Inhibitors and Inhibition Assays**—NAD synthetase inhibitors (Table 1) were synthesized as described previously, and analytical data consistent with the published data were obtained for the final purified products (31). For IC₅₀ determination, the NAD synthetase reaction was conducted as described (32) with a total volume of 50 μl in a 96-well white flat bottom 1/2-area plate. For the assay, 1 μl of compound (or solvent control) in 90% DMSO was incubated with 1 μM *M. tuberculosis* NadE enzyme in the assay buffer (50 mM Tris–HCl, pH 8.5, 10 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 50 mM KCl, 10 mM ATP, 10 mM l-glutamine) for 30 min at 37°C. The reaction was initiated by adding 10 μl of 20 mM nicotinic acid adenine dinucleotide and incubated for 60 min at 37°C. The reaction was terminated with the addition of 10 μl of stop buffer (0.3 M EDTA, 1.25 mM NaCl). Resorufin fluorescence was generated with addition of 10 μl of detection mixture (5 mM resazurin, 10 units/ml diaphorase, 0.1 M lactic acid, 100 units/ml lactate dehydrogenase, 50 mM Tris–HCl, pH 8.5) and incubated for 30 min at 37°C. Fluorescence was detected using excitation and emission filters of 560 and 590 nm, respectively.

**MIC³ determinations** were performed by the broth microdilution method (33) and the MIC was taken as the lowest concentration at which no growth was observed. Minimum bactericidal concentration was determined as the concentration that caused a >90% reduction in colony-forming units (CFU). To measure anaerobic bactericidal activity of the inhibitors, *M. tuberculosis* H37Rv was cultured in the self-generated oxygen-depletion model described in Ref. 30 using 19.5 × 145-mm tubes with a magnetic stirrer. Tubes were sealed with Teflon-lined caps and subsequently with paraplast and incubated for 3 weeks at 37°C on a magnetic stirrer. The tubes were opened in an anaerobic chamber, diluted 10-fold into anaerobic Dubos medium, and 1-ml volumes treated with various concentrations of the NAD synthetase inhibitors in 24-well plates. Control cultures were treated with DMSO. The plates were sealed in anaerobic bags and incubated for 7 days at 37°C. Serial dilutions were subsequently plated on 7H11 Middlebrook agar to monitor bacterial survival.

To measure bactericidal activity of the inhibitors against starved cultures of *M. tuberculosis*, cells were washed and resuspended at 10⁷ CFU/ml in PBST in roller bottles at 37°C. After 3 weeks of incubation, cells were treated with various concentrations of the inhibitors or DMSO vehicle control for

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3 The abbreviations used are: MIC, minimal inhibitory concentration; CFU, colony-forming unit; qRT, quantitative reverse transcription; WT, wild type.
Whole Cell Labeling with [14C]Nicotinamide—Mid-logarithmic phase cells were harvested by centrifugation and resuspended in 10 ml of 0.05 mM palmitate in minimal medium (0.5 g of casitone (Difco), 2 g of asparagine, 1 g of KH2PO4, 2.5 g of Na2HPO4, 10 mg of MgSO4·7H2O, 50 mg of ferric ammonium citrate, 0.5 mg of CaCl2, 0.1 mg of ZnSO4, 0.1 mg of CuSO4, and 0.05% Tween 80) containing 20 µCi of [14C]nicotinamide (American Radiolabeled Chemicals) to an A650 nm of 0.6. Cells were labeled for 2 days, harvested by centrifugation, and washed three times with 0.05% Tween 80 in phosphate-buffered saline (PBST). Cell pellets were extracted with 50 µl of water and 300 µl of chloroform.

For labeling of M. tuberculosis derived from infected macrophages, 105 J774 macrophages that had been infected at a multiplicity of infection of 10:1 were lysed after 2 days of infection with 0.05% SDS. Eukaryotic genomic DNA was sheared by vortexing (30 s) and M. tuberculosis was harvested by centrifugation. Cells were resuspended in minimal medium and labeled as above.

For labeling of M. tuberculosis derived from infected mouse lungs, 4-week infected mice (see below) were euthanized by cervical dislocation, and lungs were homogenized in phosphate-buffered saline (PBST) (5 ml per mouse lung) and filtered through a 40-µm filter to remove particulate material. The final volume was adjusted to 45 ml with PBST, and cells were harvested by centrifugation (2500 g, 5 min). The pellet was resuspended in 20 ml of PBST and SDS added to 0.05% final concentration to lyse eukaryotic cells. Genomic DNA was sheared by vortexing (three times for 30 s). M. tuberculosis cells were harvested by centrifugation (2500 × g, 10 min), washed once in 50 ml of PBST followed by a wash in 0.05 mM palmitate in minimal medium. Cells were labeled in 5 ml of 0.05 mM palmitate/minimal medium containing 20 µCi of [14C]nicotinamide for 24 h. Cell pellets were extracted with 100 µl of water and 300 µl of chloroform. For mouse lungs, a control uninfected mouse lung was run in parallel to ensure that no incorporation was derived from unlysed eukaryotic cells.

To label microaerophilically adapted and anaerobically adapted M. tuberculosis, M. tuberculosis was grown for 4 or 8 days into NRP-1 (microaerophilic cells) or 3 and 8 weeks into NRP-2 (anaerobic cells) (30) as described below. Tubes were briefly opened under aerobic conditions to add 20 µCi of [14C]nicotinamide after which the lids of the tubes were loosely closed and the NRP-1 tubes sealed in microaerophilic bags (type Cf), BD Biosciences) and the NRP-2 tubes in anaerobic bags (type A, BD Biosciences). An aerobic culture of M. tuberculosis of initial similar cell number (108 CFU/ml) was labeled in parallel under aerobic conditions. Cells were harvested after 3 days of incubation, washed, and extracted as above.

Analysis of 14C Nicotinamide Incorporation—Pyridine nucleotides were visualized by TLC using Whatman LHPKDF silica gel 60A plates. TLC plates were developed in 4:6 1 M ammonium acetate, pH 5, ethanol, dried, and exposed to a PhosphorImager screen (Amersham Biosciences) for 24–48 h. Migration positions of unlabeled NAD+, NADH, NADP+, NADPH, NMN, NaMN and nicotinamide standards (all from Sigma) were determined by UV shadowing.

Generation of Recombinant Proteins—The M. tuberculosis nadE gene was amplified using the primers TAGGATCCCACTTTTACTCGCTATCCAGCA and TAGGGCCGCTTAGCCCTTGGGCACCT cloned into the BamHI and NotI sites of a Gateway expression system in fusion with an N-terminal His6 tag as described (34). The protein expression was induced with 100 µM of isopropyl-β-D-galactopyranoside for 20 h at 18°C. Soluble recombinant NadE protein was purified on Nova-Gen histidine-binding column, following the recommendations of the manufacturer. The fusion protein obtained was cleaved by PreScission protease (GE Healthcare) between the His6 sequence and the N terminus of the protein following the protocol recommended by the manufacturer.

pncB1 was cloned into pET30(b)+ (Novagen) using the primers GCCATGGTGGGCCCACCCCAGCCGCC and GGATCTCTACGGGCTTGGCCGTGCA for PCR amplification (Pfx polymerase, Invitrogen) of the gene, which enabled cloning between the Ncol and BamHI sites (underlined) of the vector. Protein expression was induced by addition of 1 mM isopropyl-β-D-galactopyranoside at an A650 nm of 0.6 and induction for 3 h. Cells were lysed, and histidine-tagged protein was purified on Qiagen nickel spin columns using the native protein purification protocol recommended by the manufacturer. pncB2 was amplified using the primers CAACCATAGCGATCCGGCAGGA and GAAGCTTCTAGGTCGTTTGGCCGTGCC which enabled cloning between the Ncol and HindIII sites (underlined) of pET 28(b)+ and pET30(b)+ (Novagen). Protein expression was induced as above. Histidine-tagged protein was purified as described above. Native nonfusion protein was assayed in cell lysates prepared by sonication in 0.18 M Tris, 0.18 M potassium phosphate, pH 7.5. Control lysates were prepared from cells expressing pET28(b)+ vector.

PncB Assay—Phosphoribosyltransferase assays were performed in an assay mix consisting of 20 mM Tris, 200 mM glutamate, pH 7.4, 7 mM MgSO4, 6 mM dithiothreitol, 4 mM ATP, 0.5 mM phosphoribosyl pyrophosphate, 6 mM MgCl2, 0.017 µCi of [14C]nicotinamide or [14C]nicotinic acid and 1 µg of recombinant protein (or 10 µg of cell lysate) in a total volume of 30 µl. Reactions were incubated at 37 °C and stopped by addition of 10 µl of chloroform, and reactions were spotted onto TLC plates and developed as described above. Assays were alternatively performed in a reaction mix consisting of 30 mM potassium phosphate, 30 mM Tris, pH 7.5, 1 mM phosphoribosyl pyrophosphate, 3 mM ATP, 10 mM MgCl2, 0.017 µCi of [14C]nicotinamide or [14C]nicotinic acid, and 1 µg of recombinant protein (or 10 µg of cell lysate) in a total volume of 30 µl. Reactions were performed and analyzed as above.

Generation of Mutant Strains—A knock-out mutant of the de novo NAD biosynthetic pathway (nad::hyg) was constructed as follows. A 5074-bp Xbal-Apal cosmid DNA fragment spanning nadA–C was cloned into pcDNA2.1. A 775-bp SpHl-Asp718 fragment was replaced with the hygromycin resistance gene leaving only 775 bp of the 5' end of nadA and 1084 bp of the 3' end of nadB. A Pael fragment containing the sacB and lacZ genes from pGOAL17 (35) was cloned into the EcoRV site of
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this plasmid to generate pcnadABKO, which was used for electroporation of *M. tuberculosis*. Electroporation and generation of double crossover strains were performed as described previously (35, 36). The resulting nad::hyg knock-out strain was routinely maintained in nicotinamide-supplemented (10 µg/ml) medium. For complementation the XbaI-Apal fragment containing the entire nad–C operon along with 288 bp of upstream sequence was cloned into pMV306K (37) and electroporated into nad::hyg. Complemented mutants were selected on 7H11 Middlebrook agar supplemented with hygromycin and kanamycin but without nicotinamide.

To generate the pncB1::aph knockout mutant, a 5913-bp HindIII-BglII cosmid DNA fragment spanning pncB1 was cloned into pcDNA2.1. The aph gene was cloned into the EcoRV site of pncB1, which creates an insertion 317 bp from the start codon. A PacI fragment containing the sacB and lacZ genes from pGOAL17 (35) was cloned into the Scal site of this plasmid to generate pc1330KO, which was used for electroporation of *M. tuberculosis*. Electroporation and generation of double crossover strains were performed as above. A complementing construct was generated by cloning of a 1727-bp SphI-XmnI fragment containing pncB1 along with 250 bp of upstream sequence into pMV306H (37) and used for electroporation into the pncB1::aph strain with selection of complemented mutants on 7H11 Middlebrook agar containing hygromycin and kanamycin.

A pncB2::hyg construct was generated by cloning a 3401-bp EcoRI-Asp-718 cosmid DNA fragment containing pncB2 into pcDNA2.1. A hygromycin resistance gene was cloned into the BglII site, which generates an insertion 854 bp from the start codon. The PacI fragment from pGOAL17 was cloned into the Xbal site to generate pc0573KO, which was used for electroporation into *M. tuberculosis*. The PacI fragment containing the sacB and lacZ genes from pGOAL17 (35) was cloned into the ScaI site of this plasmid to generate pc1330KO, which was used for electroporation of this strain, and complemented mutants

Survival during Starvation—The nad::hyg mutant was grown to an *A<sub>650</sub> nm* of 0.4, the cells were washed four times with an equal volume of nicotinamide-free Dubos medium to remove extracellular nicotinamide. Cells were resuspended to an *A<sub>650</sub> nm* of 0.015 and 20 ml dispensed in 19.5 × 145-mm glass tubes containing a magnetic stirrer bar sealed as described above. Tubes were incubated at 37 °C. These cultures attained a similar *A<sub>650</sub> nm* as parallel cultures containing 10 µg/ml nicotinamide. Bacterial numbers were evaluated weekly after initiation by plating of serial dilutions onto 7H11 Middlebrook agar.

**Infection of Mice**—Lungs of 6- to 8-week-old C57Bl/6 mice were implanted with ~100–200 CFU by the aerosol route (36). Bacterial organ loads were determined by plating of serial dilutions of lung and spleen homogenates onto Middlebrook 7H11 medium from four mice per group at 0, 2, 4, 8, and 16 weeks after infection. Ten mice per group were kept to calculate median time-to-death. Mice were euthanized when they became moribund.

**Infection of Macrophages**—Bone marrow macrophages were prepared as described previously (39). Activated bone marrow macrophages were prepared by stimulation with interferon-γ and lipopolysaccharide (39). Bone marrow macrophages were infected at a multiplicity of infection of 5:1 for 3 h. Extracellular bacteria were removed by washing three times with Dulbecco’s modified Eagle’s medium. Survival was monitored by lysis of cells in 0.05% SDS and plating of serial dilutions onto Middlebrook 7H11 agar containing 10 µg/ml nicotinamide. Mycobacterial RNA for qRT-PCR analysis was prepared 1 day after infection according to Ref. 40. J774 macrophages (ATCC TIB-67) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 4 mM L-glutamine and 10% heat-inactivated fetal bovine serum, infected at an multiplicity of infection of 10:1 for 1 h, and washed three times with Dulbecco’s modified...
Eagle’s medium before adding fresh culture medium. RNA prepared 2 days after infection as above.

Measurement of NAD(H) and Menaquinone—NAD and NADH were measured from 30-ml volumes of cultures at an $A_{650\text{ μm}}$ of 0.3 using the assay described by San et al. (55) with modifications described previously (41). Dry cell weight was measured by harvesting of cells from a similar volume, transfer of cells in water to pre-weighed microtubes, and drying at 80 °C to dryness. Menaquinones and menaquinols were assayed as ratios compared with aerobic growth. M. tuberculosis during the following are shown: 1, NRP-1 hypoxic adaptation; 2, NRP-2 survival; 3, growth in J774 macrophages; 4, growth in murine bone marrow macrophages; 5, growth in activated bone marrow macrophages; 6, survival in chronically infected mouse lung.

RNA Isolation and qRT-PCR—RNA was prepared and qRT-PCR performed as described previously (36, 41). The primer and probe sets are described in supplemental Table S1.

RESULTS

Expression Analysis of the NAD Recycling Pathway under Hypoxic Conditions and during In Vivo Parasitism—The genome sequence of M. tuberculosis encodes all members of the de novo NAD(P) biosynthetic pathway (nadA, nadB, nadC, nadD, nadE, and pnpK) as well as genes required for a complete Preiss-Handler recycling pathway (pncA and the two putative pncB homologs) (Fig. 1A). The expression of all of these genes as well as that of the nadR gene encoding a probable nicotinamide mononucleotide adenylyltransferase were analyzed by qRT-PCR following infection of a mouse macrophage cell line (J774 macrophages) and infection of primary activated and unactivated mouse bone marrow macrophages, in M. tuberculosis immediately after isolation from chronically infected mouse lung tissues, under microaerophilic (NRP-1 see “Experimental Procedures”) and under anaerobic (NRP-2) conditions. Expression of the genes involved in the early steps of the de novo biosynthesis pathway was at a similar level in aerobic culture as in macrophages and under microaerophilic conditions but was slightly repressed in anaerobic culture and strongly repressed in M. tuberculosis isolated from chronically infected mice (Fig. 1B, top).

Of the genes involved in recycling, neither pncA nor pncB1 (Rv1330c) showed significant variation in expression level under these conditions. The gene encoding NadR showed a slight elevation in expression across most of these conditions, but pncB2 (Rv0573c) showed a strong up-regulation under hypoxic conditions as well as during chronic infection of mice (Fig. 1B, middle). Members of the universal pathway for NAD(P) synthesis were also repressed in anaerobic culture or from chronic murine infections (Fig. 1B), although transcript levels could be detected suggesting that the encoding proteins were still expressed at some level.

Recycling of NAD Increases during in Vivo Infection and under Hypoxia—The induction pattern of the recycling genes led us to re-examine the incorporation of radioactive nicotinamide into NAD in whole cells of M. tuberculosis under growth conditions where recycling might be favored. Previous attempts to assess this were limited to in vitro cultures under aerobic conditions, and we also found incorporation to be quite low under these conditions (Fig. 2). To test “in vivo” incorporation potential, we isolated M. tuberculosis from infected lung tissues or J774 macrophages and then labeled these bacteria with [14C]nicotinamide and examined NAD(H) by TLC after extraction. The incorporation of exogenously added nicotinamide dramatically increased during infection of macrophages and during infection of mouse lung tissues (Fig. 2, A and B, lanes 1, 6, and 7). To observe the labeling shown in lane 1, it was necessary to label 1000 times more bacteria from aerobic cul-
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In vitro assays of hexahistidine-tagged as well as native overexpressed PncB2 failed to demonstrate similar in vitro nicotinamide into NAD (Fig. 2D). This transient increase in nicotinamide incorporation into NAD during hypoxic adaptation may reflect decreased biosynthetic capacity in truly anaerobic, nonreplicating cells.

M. tuberculosis pncB1 and pncB2 Are Functional Nicotinate Phosphoribosyltransferases—PncB1 was recombinantly expressed in E. coli as an N-terminal hexahistidine-tagged fusion protein. Purified protein was assayed for nicotinate and nicotinamide phosphoribosyltransferase activity, and the radioactive products were visualized by TLC. Phosphoribosylation of nicotinic acid was observed, whereas nicotinamide could not be utilized as substrate (Fig. 3). The small amount of nicotinate mononucleotide (Fig. 3, lane 1) observed using nicotinate as a substrate (Fig. 3, lanes 1–3) was because of the conversion of a small amount of contaminating nicotinic acid present in the radiochemical (Fig. 3, lanes 2 and 3) because the nicotinic acid in the starting material was consumed and the intensity of the nicotinic acid mononucleotide spot did not further increase upon further incubation or with increased amounts of enzyme.

In vitro assays of hexahistidine-tagged as well as native overexpressed PncB2 failed to demonstrate similar in vitro nicotinamide incorporation into NAD through salvage synthesis. In vitro grown M. tuberculosis (10⁶ cells), M. tuberculosis released from infected J774 murine macrophages (5 × 10⁷ cells) were labeled with [¹⁴C]nicotinamide for 2 days (for in vitro grown M. tuberculosis) or 1 day (for in vivo grown M. tuberculosis) before analysis of pyridine nucleotides by TLC. Lane 1, WT H37Rv; lane 2, pncB1::aph; lane 3, pncB1::aph/pncB2::hyg; lane 4, nad::hyg; lane 5, pncB1::aph/pncB2::hyg/atb1::pGhsp60/pncB2; lane 6, WT from infected mouse lung; lane 7, WT from infected J774 macrophages. B, densitometric analysis of relative abundance of radiolabeled NADH (black bars) and NAD⁺ from TLC analysis. Numbering of lanes as in A. C, TLC analysis of [¹⁴C]nicotinamide incorporation into NAD (NAD⁺ not shown) by WT (lane 1) and pncB1::aph (lane 2) isolated from 4-week-old infected mouse lungs and labeled as above. D, TLC analysis of radiolabeled [¹⁴C]nicotinamide incorporation into NAD⁺ by 4 × 10⁸ aerobically growing M. tuberculosis (lane 1), 5 × 10⁷ 8-day-old microaerophilically adapted cells (lane 2), 5 × 10⁷ 3-week anaerobically adapted cells (lane 3), and 1 × 10⁸ 8-week-old anaerobically adapted M. tuberculosis (lane 4).

To assess whether the up-regulation of pncB2 observed under hypoxia (Fig. 1B) would also translate into increased activity of the salvage pathway, microaerophilically adapted or anaerobically adapted M. tuberculosis was labeled with [¹⁴C]nicotinamide under microaerophilic and anaerobic conditions (Fig. 2D). One-week microaerophilically adapted M. tuberculosis displayed strongly enhanced incorporation of [¹⁴C]nicotinamide into NAD relative to aerobically growing cells, whereas 3-week anaerobically adapted cells showed much lower levels of recycling, and 8-week anaerobically adapted M. tuberculosis failed to incorporate significant [¹⁴C]nicotinamide into NAD (Fig. 2D). This transient increase in nicotinamide incorporation into NAD during hypoxic adaptation may reflect decreased biosynthetic capacity in truly anaerobic, nonreplicating cells.
ate phosphoribosyltransferase activity for this enzyme (results not shown). Assays of PncB activity in permeabilized E. coli overexpressing native PncB2 and in permeabilized Mycobacterium smegmatis expressing PncB2 from a mycobacterial heat shock protein promoter (hs60) also failed to demonstrate any phosphoribosyltransferase activity of this protein (results not shown) above the level of the endogenous PncB activity of these organisms.

To understand the in vivo function of these two PncB homologs, we deleted pncB1 from the M. tuberculosis genome by allelic replacement with an antibiotic resistance marker. The pncB1 knock-out mutant expressed functional, albeit reduced, recycling activity as determined by incorporation of \[^{14}C\]nicotinamide into NAD (Fig. 2, lane 2). Complementation of this mutant with pncB1 expressed from the attB site restored normal levels of recycling. Deletion of both pncB1 and pncB2 resulted in a strain that was unable to incorporate exogenously added nicotinamide into NAD (Fig. 2, lane 3). Complementation of this double knock-out mutant strain with pncB2 restored recycling activity to levels comparable with the pncB1 knock-out mutant (Fig. 2, lane 5). This result demonstrates that both PncB homologs are functional, but under these conditions the activity of PncB1 is dominant.

Because of the large induction of pncB2 in chronically infected mice, we examined the contribution of this PncB homolog in animals in the M. tuberculosis strain deleted for pncB1. Similar levels of incorporation of \[^{14}C\]nicotinamide into NADH were observed between WT and pncB1::aph mutant strains isolated from infected mice, and thus PncB2 appears to be responsible for the increased salvage synthesis of NAD during infection of host tissues (Fig. 2C).

NAD Biosynthesis Is Essential for Replicating Cells of M. tuberculosis—Although exclusion from transposition had previously suggested that the early enzymes of the de novo pathway were essential, these mutants were selected under conditions not permissive for NAD salvage using nicotinamide. We therefore attempted to construct a mutant in the de novo biosynthesis pathway in the presence of exogenous nicotinamide. A genomic fragment comprising 275 bp of the 3' end of nadA and 500 bp of the 5' end of nadB was replaced with a hygromycin resistance marker. The de novo synthesis mutants obtained grew normally in the presence of nicotinamide; however, subculture of this mutant in media lacking nicotinamide resulted in a drastic reduction in CFU to below detectable levels after an initial period of normal growth for 4–6 days (Fig. 4). During this initial growth period, intracellular NAD\(^+/\)NADH ratio and levels of the oxidized and reduced pyridine nucleotides (inset) in WT as compared with NAD biosynthetic mutant strains. For x axis: 1, WT H37Rv; 2, nad::hyg in nicotinamide-replete medium; 3, nad::hyg starved for nicotinamide for 4 days; 4, pncB1::aph::pncB2::hyg; 5, WT H37Rv starved 3 days in PBST; 6, pncB1::aph. B, level of oxidized menaquinones. The x axis shows menaquinones with 8 (bar 1), 9 (bar 2), 10 (bar 3) isoprene groups and total oxidized menaquinones (bar 4). Results shown as percentage of quinone for each pair.
The decreased NAD$^+$/NADH ratio observed in the \textit{nad:hyg} mutant was also reflected in a decreased menaquinone/maenaquinol ratio (Fig. 5B), further confirming the effect of the NAD$^+$/NADH ratio on electron transport. To maintain a proton motive force during depletion of total NAD(H) levels, \textit{M. tuberculosis} would require higher levels of NADH to ensure a supply of reducing equivalents to the respiratory complexes of the membrane. Interference with various aspects of respiration has been shown previously to alter ratios of reduced and oxidized electron carriers (41). Complementation of the \textit{nad::hyg} mutant with an integrating construct, where the \textit{nadA–C} operon was expressed from the genomic \textit{attB} site, removed dependence on exogenous pyridine nucleotide precursors (data not shown).

\textbf{Interruption of NAD Synthesis Is Bactericidal for Growing Cells of \textit{M. tuberculosis}}—To explore further the consequences of sudden disruption of NAD levels, we synthesized several compounds that had been reported previously to be effective inhibitors of NAD synthetase (NadE) in several Gram-positive organisms (31). Two of these inhibitors showed modest aerobic growth inhibition at 19.5 and 17.9 $\mu$M (Table 1). The range of potencies in MIC values were paralleled in the IC$_{50}$ concentrations of the inhibitors against the purified enzyme. As expected because NadE activity is required for both recycling and \textit{de novo} pathways, the MIC values of these compounds were unaffected by the presence of exogenous pyridine nucleotide precursors such as nicotinamide (results not shown). The concentration that resulted in a greater than 1-log reduction in initial CFU (minimum bactericidal concentration) was at the MIC value for inhibitor 2 and 2-fold higher for compound 4 (Table 1). This suggests that a sudden change in the intracellular level of NAD is a lethal event. The specificity of these inhibitors for NadE was demonstrated by the time-dependent decrease in total cellular NAD levels upon treatment with these compounds. Twice the MIC and MBC concentration resulted in a 12-fold reduction in total NAD levels over 48 h with a concomitant 100-fold decrease in viable bacteria (Fig. 6A). Treatment with rifampicin at 5-fold the MIC concentration resulted in a 2.6-fold decrease in total NAD(H) even though this concentration resulted in a 97% inhibition of bacterial growth (2 $\mu$g/ml over 48 h) (Fig. 6A).

\textbf{Neither Recycling Nor \textit{de Novo} Synthesis Is Required for \textit{In Vivo} Survival}—The increased activity of the recycling pathway in \textit{M. tuberculosis} during parasitism of host cells led us to query the importance of this pathway during \textit{in vivo} pathogenesis. C57Bl/6 mice were infected by aerosol with the \textit{pncB1::aph} or \textit{pncB1::aph/pncB2::hyg} mutants, and bacterial growth in lungs and spleens was measured over time. Both mutants were able to infect and replicate similarly to wild type in host tissues (Fig. 7) indicating that NAD synthesis did not result in a measurable loss of bacterial fitness. Mice infected with the recycling mutants had a similar median time-to-death as WT-infected mice (results not shown).

To examine the metabolic plasticity of \textit{M. tuberculosis} during infection, we also infected mice with the \textit{nad::hyg} mutant strain.

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**TABLE 1**

Activities of NadE inhibitors

Summary of \textit{in vitro} activity against NadE (IC$_{50}$), MIC against H37Rv, minimum bactericidal concentration (MBC), minimum anaerobicidal concentration (MAC) defined as the concentration causing a 10-fold kill of bacteria in nonreplicating anaerobic culture after 7 days of exposure, and minimum leboecidal concentration (MLC) defined in the same way but for nonreplicating organisms under starvation conditions. In each assay, isoniazid, metronidazole, and rifampicin were included as positive controls and gave expected values. ND, not done.

| Compound Structure | IC$_{50}$ (µM) | MIC (µM) | MBC (µM) | MAC (µM) | MLC (µM) |
|--------------------|----------------|----------|----------|----------|----------|
| Insolvent          | insol          | 42 ± 11  | n.d.     | n.d.     | n.d.     |
| 24.8 ± 1.5         | 15 ± 7         | 10 ± 5   | 19 ± 12  | 39 ± 10  |
| 63.3 ± 3.0         | 155 ± 75       | n.d.     | n.d.     | n.d.     |
| 21.8 ± 1.4         | 20 ± 5         | 40 ± 10  | 42 ± 30  | > 100    |
| 57.8 ± 2.2         | 40 ± 10        | n.d.     | n.d.     | n.d.     |
Not surprisingly, the nad::hyg mutant was able to scavenge precursor for NAD synthesis from host tissues, albeit slightly less efficiently than the wild type (Fig. 7). The nad::hyg mutant replicated with similar kinetics of infection as WT-infected mice. Mice developed a chronic disease characterized by a plateau in bacterial cell numbers after 4 weeks of infection (Fig. 7) with both the de novo nad::hyg as well as the WT mutant even though total bacterial numbers were at least 10-fold less in the mutant strain. The median time-to-death of mice infected with the mutant strain was similar to that of mice infected with WT M. tuberculosis (results not shown). These results establish that either de novo synthesis or the recycling pathway are independently sufficient to support the in vivo replication of M. tuberculosis, limiting the logical choices for therapeutic targets to the enzymes of the universal pathway.

Turnover of NAD under Nonreplicating Conditions—Another important consideration for cofactor biosynthetic targets in a chronic disease such as tuberculosis is the rate of turnover of existing cofactor. In growing E. coli cells the half-life of NAD is less than 2 h, but this may vary dramatically depending upon growth conditions and metabolic flux rates (42). We therefore measured the survival of the nad::hyg mutant during starvation in PBST and under anaerobic conditions. Survival of the nad::hyg mutant in the absence of nutrients (PBST) was not affected by the presence or absence of exogenously added nicotinamide (Fig. 8A). Under anaerobic conditions the nad::hyg mutant was cultured in the presence of nicotinamide in sealed culture tubes where replication halts because of depletion of available oxygen supply (30). Anaerobic cultures were washed under anaerobic conditions to remove all available extracellular nicotinamide, and anaerobic survival was measured after 7 days in either nicotinamide-free or nicotinamide-supplemented culture medium. No difference was detected in survival between these cultures (results not shown). Because depletion of nicotinamide was followed by continued cell division under aerobic conditions, the hypoxic adaptation and survival of the nicotinamide-depleted nad::hyg strain was measured during gradual oxygen depletion in the Wayne model. There was no difference in bacterial growth or survival during hypoxic adaptation between nicotinamide-replete and nicotinamide-depleted cultures (Fig. 8B). In addition, the pncB1::aph/pncB2::hyg double mutant was able to survive at least 6 weeks under these conditions confirming the nonessentiality of the oxygen-independent salvage pathway during anaerobic persistence (Fig. 8B).

To test whether total inhibition of NAD turnover and biosynthesis through disruption of the universal pathway enzymes, which cannot be deleted genetically, lead to cell death under anaerobic conditions, the survival of anaerobically adapted M. tuberculosis during 7 days of anaerobic exposure to the NAD synthetase inhibitors was monitored. After 8 days in the self-
depleting oxygen gradient of the Wayne model, replication of \textit{M. tuberculosis} has terminated because of oxygen depletion (30) with percent oxygen saturation lower than 1%, whereas at 14 days the dissolved oxygen concentration is lower than 0.06% (30). Cells from 8-, 14-, and 21-day adapted Wayne model cultures remained sensitive to inhibitors 2 and 4, with inhibitor 2 at 19 \( \mu \text{M} \) resulting in a greater than 1-log reduction in bacterial number (Table 1). In all of these anaerobic assays, isoniazid, an antitubercular drug that is ineffective under anaerobic conditions (30), resulted in no measurable reduction in bacterial numbers at concentrations 10-fold higher than its MIC, whereas metronidazole, as expected (30), resulted in a 2-log reduction in bacterial numbers at 100 \( \mu \text{M} \) (Table 1). Measurement of cellular NAD levels showed that the inhibitors resulted in a greater depletion of this cofactor over time than in untreated anaerobic cells (Fig. 6B). The effect of inhibitors 2 and 4 was also tested against \textit{M. tuberculosis} that had been starved for nutrients in PBST which showed that inhibitors 2 and 4 resulted in a 99% reduction in CFU at 78 and 140 \( \mu \text{M} \), respectively (Table 1).

**DISCUSSION**

NAD is a ubiquitous cofactor, and several proteins involved in its synthesis have been proposed as potential drug targets (43–46). NAD can be obtained by intracellular pathogens either through \textit{de novo} synthesis or by parasitism of host niacin incorporated through a recycling pathway. In \textit{M. tuberculosis}, this recycling pathway was previously thought to be nonfunctional (13, 26, 43) despite the presence of two putative \textit{pncB} homologs in the \textit{M. tuberculosis} genome (19). We have demonstrated that NAD scavenging from host tissues is highly induced during \textit{in vivo} growth and upon exposure to low oxygen conditions. Earlier reports where this pathway was reported to be nonfunctional were based upon the very low levels of incorporation of exogenous nicotinamide in aerobically replicating cells, a finding we have confirmed.

A Preiss-Handler-independent pathway could be envisaged by the activity of NadR on pyridine nucleotides or the activity of a nicotinamide phosphoribosyltransferase in combination with NadR on nicotinamide as substrate. A gene with homology to known pyridine nucleotide transporters has not been identified in the \textit{M. tuberculosis} genome. In addition \textit{M. tuberculosis} appears to lack a \textit{nadV} homolog. Alignment of \textit{M. tuberculosis} NadR to the NadR proteins of \textit{E. coli}, \textit{Salmonella typhimurium}, and \textit{Haemophilus influenzae} revealed that the ribosyl nicotinamide kinase domain was possibly nonfunctional because of mutation of several residues that had been found to be located in the active site of the \textit{H. influenzae} NadR crystal structure. This includes a mutation in the Walker-A or P-loop (GGESSGKS mutated to GPSSGKS) and the absence of a Walker-B (IAFID) motif. The NMNAT domain shares all residues predicted to interact with the substrate except for a conserved glutamate residue in the nicotinamide recognition domain, which is mutated to a proline residue in \textit{M. tuberculosis} NadR.

Surprisingly, both the \textit{pncB} homologs contribute to the Preiss-Handler pathway because salvage from nicotinamide was only abolished by the combined deletion of both genes (summarized in Table 2). Alignment of these enzymes with known nicotinic acid phosphoribosyltransferases, including the two crystallized enzymes from yeast and from \textit{Thermoplasma}, showed that residues that interact with nicotinic acid, phosphoribosyl pyrophosphate, and with AMP, as well as residues that were implicated in catalysis (47, 48), were conserved in both (supplemental Fig. S1A). In addition, the histidine residue involved in autophosphorylation is conserved among all these PncB homologs. Post-translational modification may contribute to the catalytic efficiency of members of this family, for example phosphorylation of the \textit{Salmonella} PncB converts the enzyme to a high affinity form with a high turnover (49). It is possible that such a post-translational modification is required for enzymatic activity of both PncB homologs because we could only demonstrate \textit{in vitro} activity of one (PncB1). Nonetheless, our results support a role for both enzymes. PncB1 appears to be unregulated by growth conditions but contributes to basal NAD levels, whereas PncB2 appears to be specifically regulated by \textit{in vivo} growth and hypoxia (Table 2). Both enzymes appear to be specific for nicotinic acid as opposed to nicotinamide, and this finding is supported by the close alignment of PncB2 with

**FIGURE 8.** \textit{De novo} NAD biosynthesis is not required during nonreplicating persistence. \textbf{A}, analysis of survival during starvation of the \textit{nad:hhyg} mutant in PBST in the presence (●) and absence (224) of nicotinamide (Nam); \textbf{B}, survival of \textit{NAD} biosynthetic mutants during adaptation and survival in hypoxic conditions. ●, \textit{nad:hhyg} in the presence of nicotinamide; 224, \textit{nad:hhyg} in the absence of nicotinamide; ○, WT H37Rv; ▲, \textit{pncB1:aph/pncB2:hhyg}; ▼, WT.
known nicotinic acid phosphoribosyltransferases as opposed to nicotinamide phosphoribosyltransferases encoded by nadV genes (supplemental Fig. S1B). Moreover, the pncB1::aph mutant was able to convert [14C]nicotinic acid to [14C]NAD (results not shown) further demonstrating that the substrate for PncB2 is also the acid and not the amide. The presence of two pncB genes in the M. tuberculosis genome is an intriguing ridge. Mycobacterium leprae with its extensive gene deletion has lost all the components of the recycling pathway. M. leprae, however, occupies a very different niche within the host than M. tuberculosis and has lost the ability to grow axenically. In M. smegmatis, only one pncB gene, with higher homology to pncB1, is present in the genome. In contrast, the sporulating actinomycete Streptomyces coelicolor possesses two pncB homologs (50).

Thus PncB2 appears to be more important in the adaptation to nonreplicating persistence. Various in vitro models have been developed to reproduce the environmental signals that result in a switch to nonreplicating persistence, including hypoxia (30, 51), nitric oxide (52), and starvation (53, 54). The up-regulation of pncB2 expression during hypoxia and increased activity of the salvage pathway under low oxygen concentrations suggest an important role of NAD salvage during adaptation to microaerophilic conditions as would be expected to occur in human granulomas (3). In fact, pncB2 has been reported previously to be a member of the DosR regulon, the suite of genes engaged when M. tuberculosis is exposed to hypoxic conditions (52). The increased level of NAD salvage observed in microaerophilically adapted cells indicates that transcript levels of pncB2 could be correlated with activity of the salvage pathway. Thus, DosR mediates a switch from de novo synthesis to salvage of NAD from host nicin consistent with the observed down-regulation of expression of the genes of the de novo pathway (nadA–C) during parasitism of host tissues. The increased activity of recycling enzymes in M. tuberculosis isolated from mouse tissues also corroborated this notion. In addition, the similar levels of [14C]nicotinamide incorporation into NAD between WT and pncB1::aph strains isolated from infected mouse lungs indicated that the increased transcript levels of pncB2 were directly responsible for the increase in recycling activity.

The pncB1::aph/pncB2::hyg mutant, however, displayed no difference in survival under in vitro microaerophilic and anaerobic conditions (results not shown). The survival of this mutant under anaerobic conditions was surprising because the de novo pathway is oxygen-dependent. The result is consistent with the “burst” of [14C]nicotinamide labeling seen in anaerobically adapting cells, suggesting that the major requirement for NAD may be during the transition to anaerobiosis. These results suggest that NAD is turned over extremely slowly under these nonreplicating conditions. However, the in vitro hypoxia model is highly stringent with oxygen concentrations approaching true anaerobiosis, whereas measurement of actual oxygen tension in live infected rabbit lesions suggests that the oxygen tension during disease is 1.6 ± 0.7 mm Hg (51).

The phagosomal environment appears to be nutrient-deficient (3, 40), and thus the switch to salvage synthesis probably reflects the conservation of metabolic energy in an environment that is restrictive for nutrients. The ability of a de novo NAD biosynthetic pathway mutant to infect, replicate, and persist in mouse tissues, albeit at lower levels than the parental WT strain, demonstrates that salvage synthesis can support in vivo pathogenesis. This simple observation illustrates another major problem in selection of targets for chemotherapeutic intervention because targets that may appear to be essential in vitro may in fact be dispensable in vivo. In this case the de novo NAD biosynthetic pathway, which appeared to be essential in an in vitro genome-wide mutagenesis screen (20), was established as nonessential in vivo because of the abundance of niacin (nicotinamide and nicotinic acid) in the host.

It remains to be established whether recycling is of greater importance in other animal models or in infected human hosts. However, knock-out of both pncB2 and pncB1 in M. tuberculosis did not result in any defect in parasitism of mouse lungs and spleen, suggesting that, at least in this animal model, the de novo biosynthetic pathway can replace salvage synthesis without apparent loss of fitness.

Our results do demonstrate that inhibition of both pathways for NAD synthesis resulted in cell death, which is obviously a desirable outcome for chemotherapeutic intervention. This was evidenced by two important observations as follows: 1) the rapid loss of viability of the nad::hyg mutant in the absence of exogenous nicotinamide, and 2) the observation of the same effect using the indole derivatives previously described as NadE inhibitors. The nad::hyg mutant was unable to support the same levels of NAD(H) synthesis as its WT parental counterpart and as a result displayed an increased NADH:NAD+ ratio as well as an increased menaquinol:menaquinone ratio. This suggests an important knock-on effect of rapidly reducing total NAD(H) levels on cellular respiration. Microarray analysis of RNA isolated from M. tuberculosis during treatment with the NadE

### Table 2

**Phenotypes of nicotinamide biosynthesis and recycling mutants**

Summary of the differences in phenotypes observed with the Mtb mutants used in this study. ND, not done.

|                           | WT            | nad::hyg      | pncB1::aph    | pncB1::aph/pncB2::hyg |
|---------------------------|---------------|---------------|---------------|-----------------------|
| [14C]Nicotinamide incorporation | +            | +             | +             | –                     |
| [14C]Nicotinic acid incorporation | +            | +             | +             | –                     |
| Survival (nicotinamide starvation) | +           | –             | +             | –                     |
| Total NAD(H) in nmol/mg in vivo | 1.03 ± 0.18  | 0.18 ± 0.03*  | 0.86 ± 0.12   | 0.93 ± 0.22           |
| Fold increase in vivo/in vitro | 100–1000×    | ND            | 6 × 10³       | 2.0 × 10³             |
| Phenotype in vivo          | 3.1 × 10⁵     | 100–1000      | 0.12          | 1.3 × 10⁵             |

*Total NAD(H) after 4 days of nicotinamide starvation.

**Table notes**
- ND: not done.
- *: Significant difference compared to WT.
inhibitors demonstrated that the transcriptional profiles elicited by these compounds were similar to those induced by respiratory inhibitors (results not shown).

The indole derivatives are substrate analogs with two aromatic groups linked by an aliphatic chain meant to mimic the pyridine nucleotide and adenine of nicotinic acid adenine dinucleotide, and have been reported previously to have IC50 values of 9 μM to >100 μM against the Bacillus subtilis NadE enzyme (31). These compounds had also demonstrated MIC values ranging from 1.5 μM to >50 μM for several Gram-positive organisms including B. subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, and Streptococcus enteritidis. These molecules inhibited the purified M. tuberculosis enzyme in vitro, and the two inhibitors with the lowest IC50 values displayed MICs of 15–20 μM against M. tuberculosis grown in vitro. These two inhibitors were bactericidal against M. tuberculosis after 7 days of treatment in vitro corroborating the phenotype of the nadCΔhyg mutant. The bactericidal effect of abrogation of NAD synthesis suggests that treatment of M. tuberculosis actively growing in host tissues with inhibitors of this pathway will lead to eradication of the organism. Because of considerable cytotoxicity of the NAD synthetase inhibitors against eukaryotic cell lines (results not shown), the effect of these compounds against M. tuberculosis residing in infected macrophages could not be tested.

The bactericidal activity of the NAD synthetase inhibitors on nonreplicating bacteria persisting under anaerobic conditions and in nutrient-starved cultures suggests that inhibition of this pathway may be a useful strategy for either treating latent infection or shortening the duration of TB chemotherapy. In summary, these studies establish that NAD biosynthesis is an attractive drug target for actively replicating as well as nonreplicating M. tuberculosis. The targets are, however, limited to enzymes within the universal pathway because of the presence of both a de novo and a salvage pathway for pyridine nucleotide synthesis. These studies highlight the biological uncertainty faced during target selection based upon the fundamental differences in the questions of genetic essentiality compared with the nature of chemical interruption of function represented by addition of a drug, particularly in the context of a chronic disease. Selection of targets for drug development therefore depends on an understanding of the metabolic importance of the protein during in vivo pathogenesis and the network of genetic pathways available that the organism may engage to avoid a bactericidal effect. Genetic experiments rarely duplicate the kinetics or extent of interruption of function achieved by a targeted small molecule, making the intrinsic value of a target very difficult to assess. In a chronic disease such as tuberculosis, these problems are particularly acute because treatment is limited to an established infection, thus biological uncertainty in target selection is a major impediment to the development of new therapies. The NAD biosynthetic pathway offers many important lessons in the depth of understanding of a metabolic pathway necessary to effectively select a potential target.

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