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Evaluation of DNA Primase DnaG as a Potential Target for Antibiotics

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Mycobacteria contain genes for several DNA-dependent RNA primases, including dnaG, which encodes an essential replication enzyme that has been proposed as a target for antituberculosis compounds. An in silico analysis revealed that mycobacteria also possess archaeo-eukaryotic superfamily primases (AEPs) of unknown function. Using a homologous recombination system, we obtained direct evidence that wild-type dnaG cannot be deleted from the chromosome of Mycobacterium smegmatis without disrupting viability, even in backgrounds in which mycobacterial AEPs are overexpressed. In contrast, single-deletion AEP mutants or mutants defective for all four identified M. smegmatis AEP genes did not exhibit growth defects under standard laboratory conditions. Deletion of native dnaG in M. smegmatis was tolerated only after the integration of an extra intact copy of the M. smegmatis or Mycobacterium tuberculosis dnaG gene, under the control of chemically inducible promoters, into the attB site of the chromosome. M. tuberculosis and M. smegmatis DnaG proteins were overproduced and purified, and their primase activities were confirmed using radioactive RNA synthesis assays. The enzymes appeared to be sensitive to known inhibitors (suramin and doxorubicin) of DnaG. Notably, M. smegmatis bacilli appeared to be sensitive to doxorubicin and resistant to suramin. The growth and survival of conditional mutant mycobacterial strains in which DnaG was significantly depleted were only slightly affected under standard laboratory conditions. Thus, although DnaG is essential for mycobacterial viability, only low levels of protein are required for growth. This suggests that very efficient inhibition of enzyme activity would be required for mycobacterial DnaG to be useful as an antibiotic target.

Mycobacterium tuberculosis is a deadly pathogen that claims nearly 2 million lives annually and infects an estimated 2 billion people, who serve as a reservoir of latently infected individuals (1). Most tuberculosis (TB) cases are not the result of new infections but are caused by the reactivation of dormant M. tuberculosis (2). TB caused by drug-sensitive strains is fully treatable, but patients must take three or four drugs for approximately 6 months. Premature termination of drug therapy results in the emergence of resistant strains. The World Health Organization estimates that 50 million individuals harbor multidrug-resistant (MDR) M. tuberculosis, which is resistant to at least rifampin and isoniazid. Treating these MDR strains requires second-line drugs, which are expensive, have side effects, and take longer to work (up to 2 years). More disturbing is that strains of untreatable extensively drug-resistant (XDR) TB, which are additionally resistant to any fluoroquinolone and at least one of three injectable second-line drugs (capreomycin, kanamycin, or amikacin), have already been identified in 58 countries. This XDR form, together with totally drug-resistant (TDR) TB, seems to represent the greatest health threat (3). The options for treating MDR/XDR/TDR TB infections are becoming seriously limited, threatening to return TB control to the preantibiotic era (4, 5). The first-line drugs for treating TB are restricted to a few sensitive targets, including inhA (NADH-dependent enoyl-[acyl carrier protein] reductase) and kasA (3-oxoacyl-[acyl carrier protein] synthase 1) for isoniazid, rpoB (DNA-directed RNA polymerase subunit beta) for rifampin, and the embCAB operon for ethambutol. Also in this category are enzymes required for the intracellular activation of currently used drugs, such as katG (catalase peroxidase peroxynitrite T) for isoniazid, pncA (pyrazinamidase/nicotinamidase) for pyrazinamide, and etaA (monooxygenase) for ethionamide (6). The identification of new drugs and sensitive targets would appear to be indispensable for the control of drug-resistant forms of TB. One requirement for a promising antibacterial enzyme target is that it be essential for the organism and that it not be present in the host. Such candidates might be found among basic essential metabolism pathways, including DNA replication processes.

Bacterial DNA replication is performed by PolIII, which is unable to synthesize DNA de novo and therefore requires a primer to allow the initiation of DNA synthesis. The replication of leading strands requires at least a single primer to initiate the process, but replication of the lagging strand requires an individual starter for each Okazaki fragment. In Escherichia coli, the enzyme that synthesizes such primers is the RNA polymerase, DnaG. Eukaryotes also possess a distinct primase responsible for the synthesis of RNA primers. DNA primase is a single-strand DNA (ssDNA)-dependent RNA polymerase that plays a key role in DNA synthesis (7). The DNA primases of bacteria and bacteriophages are classified into one group, and the primases of eukaryotes and archaea belong to a second group. All primases share many catalytic prop-

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erties, but the proteins in the two classes differ both in structure and in their relationship with other proteins in the replication complex (8, 9). The prokaryotic primase associates with the replicative DNA helicase. DnaG primase contains three distinct domains, an N-terminal zinc-binding region, a middle RNA polymerase domain, and a C-terminal domain containing either a DNA helicase (phage) or a region for interaction with the DNA helicase (bacteria) (10). In contrast to DnaG primase, which is monomeric, eukaryotic primase is a heterodimeric complex of DNA polymerase α and an accessory β subunit. The small primase subunit (PriS) belongs to the archaeo-eukaryotic primase (AEP) superfamily (11). The PriS complex contains an active site for RNA primer synthesis and the large primase accessory subunit (PriL), which may coordinate primase and polymerase action and is required for the initiation of primer synthesis (12). Previous studies have demonstrated that AEPs are also present in diverse bacteria (13, 14).

An AEP domain constitutes one of three domains in ATP-dependent ligase (LigD), which is a key protein in the nonhomologous end-joining (NHEJ) DNA repair system (11, 15, 16, 17, 18). The primase domain has terminal transferase, DNA-dependent RNA primase, and DNA-dependent DNA/RNA gap-filling polymerase activities (15, 16, 18, 19, 20). In mycobacteria, both DnaG and AEPs have been reported. The replicative DnaG primase is encoded by the dnaG gene, which is located in the dnaG operon (21).

The viability of DnaG primases as antibiotic targets rests on the presumption that these enzymes are essential for all bacteria because they are required for initiating DNA replication. However, it is difficult to definitively establish this indispensability, which is a fundamental prerequisite if these enzymes are to be considered potential antibiotic targets. In this report, we undertook a series of experiments that unequivocally demonstrate that dnaG is essential in Mycobacterium smegmatis, even in AEP-overexpressing backgrounds. We also characterized the enzymatic activities of M. smegmatis and M. tuberculosis DnaG proteins. A detailed analysis of the amount of DnaG in various strains revealed that the level of protein can vary by ~6-fold without producing a major effect on growth under standard laboratory conditions. Strains engineered during this study will be useful in any future detailed evaluation of antibiotics targeting DnaG.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study were derived from M. smegmatis mc²155 (22) and were cultured in Middlebrook 7H9 broth supplemented with albumin-dextrose-sodium chloride or NB broth (8.0 g/liter nutrient broth [Difco], 10.0 g/liter glucose). Where required, further additions included 0.2% Tween 80 (pH 6.0 to 6.2), 50 μg/ml hygromycin (Hyg), 7.5 μg/ml gentamicin (Gen), and 25 μg/ml kanamycin (Kan). Mycobacterial transformants were selected on Middlebrook 7H10 agar plates enriched with albumin-dextrose-sodium chloride containing Kan (25 μg/ml), Gen (7.5 μg/ml), or Hyg (50 μg/ml). E. coli strains were cultured in LB medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl, pH 7.0). Where required, further additions included 100 μg/ml ampicillin (Amp), 200 μg/ml Hyg, and 50 μg/ml Kan.

Gene-cloning strategies. Standard molecular biology methods were used for all cloning protocols (23). All PCR products were obtained using thermostable AccuPrime Pfx DNA polymerase (Invitrogen), cloned initially into a blunt vector (pBluescript II; Fermentas), sequenced, and then released by digestion with the appropriate restriction enzymes before cloning into the final vectors. Subcloning into expression vectors was facilitated by incorporating restriction enzyme recognition sites into the sequence of the primers. The plasmids used in this work are listed in Table S1 in the supplemental material.

Construction of gene-replacement vectors. To perform unmarked deletions of dnaG, prim2, prim3, and prim4 genes in M. smegmatis, we used a suicidal recombination delivery vector based on p2NIL (24). The recombination vector carried the 5’ end of dnaG with the upstream region connected to the 3’ end of the gene with the downstream region, amplified with the primers shown in Table S2 in the supplemental material. The accC1 gene was then introduced between the upstream and downstream regions of dnaG. Finally, the Pael screening cassette from pGOAL17 was inserted into the constructs, resulting in the suicide delivery vectors pMK165, pBAl48, pMK189, and pMK190 carrying ∆dnaG::aacC1?, ∆prim2, ∆prim3, and ∆prim4, respectively; these were used to engineer the directed M. smegmatis mutants as described previously (25, 26). The resultant mutant strains were verified by PCR and Southern blot hybridization (see Fig. S1 in the supplemental material).

Construction of complementation plasmids. M. smegmatis genes (dnaG, ligD, prim2, prim3, prim4), M. tuberculosis dnaG, and E. coli dnaG were amplified by PCR using the primers listed in Table S2 in the supplemental material and cloned into the BamHI-XbaI sites of the pBluescript vector downstream from the Ppap promoter (see Table S2). Next, all genes with their Ppap promoters were excised from these vectors with HindIII and XbaI and cloned into the integration vector pMV306, generating pMK172, pMK207, pBAl45, pMK206, pMK193, pMK173, and pMK174 for dnaG, ligD, prim2, prim3, prim4 (M. smegmatis), dnaG (M. tuberculosis), and dnaG (E. coli), respectively. The M. smegmatis dnaG gene was amplified by PCR using the primers listed in Table S2 and cloned into the BamHI-HindIII sites of the pKW08lux vector downstream from the Plux promoter (see Table S2). Next, genes with their Plux promoters were excised from this vector (pMK214) with HindIII and XbaI and cloned into the integration vector pKW08lux-Int, generating the pMK215 vector.

Disruption of M. smegmatis genes encoding primases. The protocol of Parish and Stoker (24) was used to disrupt the M. smegmatis dnaG prim2, prim3, prim4, and ligD genes at their native loci on the chromosome. The mutants were generated by subsequent disruption of individual genes. The suicidal recombination plasmid DNA (pMK165, pBAl48, pMK189, pMK190, and pMK111) was treated with NaOH (0.2 mM) and integrated into the M. smegmatis mc²155 chromosome by homologous recombination. The resulting single-crossover recombinant (SCO) mutant colonies were blue, resistant to Kan and Gen, and sensitive to sucrose. The site of recombination was confirmed by PCR and Southern hybridization. The SCO strains were further processed to select for double-crossover (DCO) mutants that were white, sensitive to Kan, and resistant to sucrose (2%). PCR and Southern hybridization analyses were used to distinguish between the wild type and each mutant DCO. Probes that hybridized to each gene, labeled using a nonradioactive primer extension system (DIG-labeling system; Amersham, GE Healthcare, Sweden), were generated by PCR. The primers used for PCR amplification are listed in Table S2 in the supplemental material.

Protein purification. For protein expression, all E. coli cultures were grown at 37°C in LB medium containing Amp. The expression constructs were made by PCR amplification of M. smegmatis dnaG (dnaG_Ms), and M. tuberculosis dnaG (dnaG_Mt), cloning into pBluescript II, excising with BamHI/HindIII (for dnaG_Ms) or BamHI/EcoRI (for dnaG_Mt), and cloning into the final vectors, pPHS and pGEX, respectively. The pPHS and pGEX derivatives were transformed into E. coli BL21(DE3), and cells were plated on LB agar containing antibiotics and grown overnight. Single colonies were inoculated into 5 ml liquid medium, grown overnight, and diluted 100-fold into fresh medium (300 ml). After the colonies were grown to midexponential phase (optical density at 600 nm [OD₆₀₀] of 0.8 to 1.0), protein expression was induced by the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside) to 0.4 mM. After overnight incubation, cells were harvested, sonicated, and centrifuged to separate the soluble and insoluble fractions. DnaG DNA primase was purified from the soluble
fraction by affinity column chromatography using Ni²⁺-charged His
Bind resin (Novagen) for the His-tagged protein and glutathione agarose
(Pierce) for the glutathione S-transferase (GST)-tagged protein. After
concentration of protein using Amicon Ultra 4-mL concentrators with a
30,000 molecular-weight cut-off polyethersulfone (PES) membrane, pro-
tein sample concentrations were determined using the bicinchoninic acid
(BCA) method (Bio-Rad protein assay). The purified DnaG and LigD (15)
were used for rabbit immunization as described previously (26).

DnaG primase activity assay. In vitro assays of primase activity were
performed essentially as described previously (27). The reaction mixture
(total volume, 25 μL) for the RNA primer synthesis assays contained 50
mM HEPES (pH 7.5), 100 mM potassium glutamate, 10 mM dithiothre-
tol, 10 mM magnesium acetate (or 10 mM manganese chloride), and 2
μM ssDNA template (5′-taacctcatggaactccgacga). DnaG primase was
added to a final concentration of 3 μM, and the sample was preincubated
for 10 min at 30°C. After the addition of ATP, CTP, GTP (each to a final
concentration of 200 μM), UTP (to a final concentration of 20 μM), and
0.6 μCi of [α-32P]UTP (3,000 Ci/mmol), the reaction was incubated for an
additional 4 h. The reaction was stopped by adding 30 μL of 3 M sodium
acetate. The RNA products were then precipitated overnight at −70°C
with 96% cold ethanol in the presence of 40 μg glycogen. The precipitates
were washed with 75% cold ethanol and dissolved in 8 μL loading buffer
(95% formaldehyde, 0.05% bromophenol blue, 0.05% xylene cyanol, 20
mM EDTA). Samples were heated at 98°C for 10 min, and the reaction
products were separated by electrophoresis in 18% urea-polyacrylamide
gel. After electrophoresis, the results were visualized by autoradiography
using X-ray film with intensifying screens overnight at −70°C. The sensi-
tivity of DnaG to inhibitors (suramin and doxorubicin) relative to the
controls was determined by adding an inhibitor to the reaction buffer at
concentrations ranging from 1 to 100 μM.

AlamarBlue and CFU susceptibility tests. The microplate alama-
Blue assay (28) was used to test the sensitivity of mycobacteria to DnaG
inhibitors. Suramin and doxorubicin were dissolved in NB medium at
final concentrations of 0.039 to 5 mM (suramin) or 0.98 to 125 μM (doxo-
rubicin), filtered (0.22 μm), and distributed in the wells of microtiter
plates (100 μL/well). Next, 100 μL of wild-type M. smegmatis and comple-
mented ΔdnaG mutants (OD600, 0.1) was added to the control wells and
to the wells containing inhibitors. The plates were incubated at 37°C for 72
h in a humidified atmosphere. AlamarBlue reagent (25 μL; Invitrogen)
was added, and the plates were incubated overnight at 37°C. A color
change from blue to pink indicated bacterial growth. The MIC was de-
finned as the lowest drug concentration that prevented a color change.
The sensitivity of mycobacteria to suramin and doxorubicin was also deter-
mined by monitoring the numbers of CFU of the wild-type and mutant
strains growing in the presence of different concentration of inhibitors as
described previously (29).

RNA extraction and reverse transcription. For quantitative real-time
PCR (qRT-PCR) experiments with prim2, prim3, and prim4 transcripts,
RNA was extracted from wild-type M. smegmatis strains and from the
SCO strains (ΔdnaG/CaaacC1-dnaG) carrying an extra copy of an AEP gene
under the control of a chemically inducible promoter (attB-P-pol prim2/prim3/prim4) using the TRIZol LS reagent (Invitrogen) as described pre-
viously (26). For reverse transcription, we used a SuperScript III first-
strand synthesis system (Invitrogen) and performed the reactions in total
volumes of 20 μL containing 1 μg of total RNA. Subsequently, 2 μL of
cDNA (equivalent to 50 ng of RNA) was used in the qRT-PCR experi-
ments (see below).

qRT-PCR. qRT-PCR for the analysis of prim2, prim3, and prim4 gene
expressions was performed using the Maxima SYBR green qPCR master
mix (Fermentas) and a 7900HT real-time PCR system (Applied Biosys-
tems). Each reaction mixture (final volume, 25 μL) was mixed on ice
and contained 1X Maxima SYBR green qPCR master mix, 50 ng of cDNA, and
0.3 μM each primer (see Table S2 in the supplemental material for primer
sequences). For expression analysis of the M. smegmatis prim2, prim3, and
prim4 genes, we used a three-step cycling protocol in which the reaction
mixtures were first heated to 95°C for 10 min and were then subjected to
45 cycles at 95°C for 15 s (denaturation), at 63°C for 30 s (annealing), and
at 72°C for 30 s (extension). The data were acquired during the extension
step. To verify the specificities and identities of the PCR products gen-
erated, melting curve analysis was performed at the end of each PCR. Each
experiment was performed in triplicate, and the results are presented as
the means and standard errors. The results were normalized with respect
to sigA gene expression as the internal control and reflect the fold change
in the expression of a given gene in the mutant strain versus the wild-type
strain, as calculated using the double-delta method 2^ΔΔCT (30).

RESULTS

Rv2343c and Msmeg4482 genes encode active primases that are
sensitive to doxorubicin and suramin. Bacterial primases (DnaG) are highly
conserved across all bacterial genomes, allowing ready identification of homologous genes by bioinformatics
analysis. The DnaG of M. tuberculosis displays 48% similarity and
32% identity with its E. coli counterpart and as much as 89% similarity and 82% identity with the DnaG of M. smegmatis (see
http://www.ncbi.nlm.nih.gov/BLAST/). In contrast, bacte-
rial primases are distinct from their eukaryotic and archaeal counter-
parts, which makes them an attractive antibacterial drug target
(31, 32). The genomes of fast- and slow-growing mycobacteria
each carry a single gene that is homologous to bacterial dnaG
(Msmeg4482 and Rv2343c for fast and slow growers, respec-
tively). The activity of M. tuberculosis DnaG was recently con-
firmed in an in vitro study (32). We used a pT7 Pol-based E. coli
cDNA expression system to overproduce and purify DnaG from
M. smegmatis and M. tuberculosis, and we subsequently used the pu-
rified DnaG of M. smegmatis for the vaccination of a rabbit to
obtain polyclonal antibodies. The primase activities of DnaGs
from these two strains were monitored by visualizing and quanti-
fying radiolabeled RNA primer products on denaturing gels. We
tested a number of templates and reaction parameters to identify
the optimal conditions for DnaG activity. A reaction buffer sup-
plemented with Mn²⁺ or Mg²⁺ and a 24-mer (5′-taacctcatggaactccgacga) ssDNA template allowed us to confirm the activity of
both enzymes (Fig. 1). DnaG activity was suppressed in the pres-
ence of doxorubicin (100 μM) and suramin (10 μM), known
inhibitors of DnaG (32), which inhibited the activities of M. smeg-
matis and M. tuberculosis DnaG in vitro by about 85% and 70%,
respectively (Fig. 2A). Notably, doxorubicin, but not suramin,
displayed antibacterial activity against M. smegmatis grown in liq-
uid culture. The alamarBlue assay, which allowed us to monitor
cell viability, showed that 8 μM doxorubicin inhibits the growth of
mycobacteria. On the other hand, suramin was tolerated even at
concentrations of 5 mM (see Fig. S2 in the supplemental material).
The CFU analysis of M. smegmatis growing in the presence of
different concentrations of doxorubicin revealed dose-dependent
inhibition of growth (Fig. 2B). Moreover, the M. smegmatis mu-
tant expressing as little as about 20% of the physiological level of
DnaG appeared to be more sensitive to doxorubicin than the wild-
type strain (Fig. 2C). In the presence of 2.5 μM and 3 μM doxo-
rubicin, the numbers of viable cells were at 50% and 20%,
respectively, of those viable in the wild-type culture.

The activity of DnaG, but not that of primases in the AEP
family, is essential for M. smegmatis viability. Bioinformatic
analyses of the genomes of M. tuberculosis and M. smegmatis
showed that, in addition to dnaG, mycobacteria also contain pu-
tative AEP-like primase genes. The best characterized of these is
PolDom, an AEP that is part of a multidomain enzyme called

DnaG as a Putative Target for New Tuberculostatics

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ligase D (LigD), which is required for NHEJ DNA repair during the stationary phase (15,19). The function of other genes displaying homology to AEPs remains to be elucidated. Similar to the case in other bacteria, the *dnaG* gene of *M. smegmatis* was previously reported to be essential for viability (21). Here, we used a gene-replacement strategy to verify that *dnaG* is essential in mycobacteria and tested whether AEPs are also essential. In contrast to *dnaG*, which could not be replaced by a nonfunctional copy without disrupting viability, individual or collective removal of intact AEP genes in *M. smegmatis* to generate /H9004 MSMEG_5570, /H9004 MSMEG_6301, /H9004 MSMEG_0597, /H9004 MSMEG_2105 or /H9004 (MSMEG_5570; MSMEG_6301; MSMEG_0597; MSMEG_2105) was well tolerated, establishing that the AEPs are not essential for the viability of *M. smegmatis* (see Fig. S1 in the supplemental material). To further confirm that *dnaG* is essential and to engineer a *dnaG* conditional mutant, we cloned the intact gene into a plasmid that placed it under the control of a tetracycline- or acetamide-inducible promoter (Ptet/Pami) and introduced this construct into the *attB* locus of *M. smegmatis* chromosomal DNA. The additional *dnaG* copy enabled us to replace the native *dnaG* gene with its mutated copy. The genotype of each mutant was confirmed by Southern blotting hybridization (Fig. 3). Additionally, the *attB* integrated, intact copy of *dnaG* provided with the pMV306Hyg′ vector was subjected for replacement with an “empty” pMV306Kan′ vector in six independent experiments. The lack of Kan′ recombinants without an intact *dnaG* confirmed additionally the essentiality of DnaG for viability of *M. smegmatis* (33).

DnaG is not complemented by AEPs. Since the only essential primase in mycobacteria is DnaG, it can be considered essential for replication of chromosomal DNA. To evaluate DnaG as a potential drug target, we tested whether it is still essential in an AEP-overexpressing background. Genes encoding AEPs were cloned to place them under the control of a chemically inducible promoter (P<sub>amr</sub>) and integrated into the *attB* locus of an *M. smegmatis* SCO mutant carrying both a functional *dnaG* gene and a *dnaG* gene disrupted by the aacC1<sup>R</sup> gene (/H9004 *dnaG*::aacC1<sup>R</sup>). The overexpression of AEPs was confirmed by Western blot analysis (LigD) or quantitative RT-PCR (prim2, prim3, and prim4) (see Fig. S3 in the supplemental material). Next, we selected for mutants lacking an intact copy of *dnaG* (i.e., those carrying Δ*dnaG::aacC1<sup>R</sup>* exclusively). In no case did the overexpression of AEPs rescue the viability defect of *M. smegmatis* lacking an intact chromosomal *dnaG* gene, confirming that *dnaG* is essential and demonstrating that these primases serve nonredundant functions. Next, we tested whether *M. smegmatis* *dnaG* could be replaced by its counterpart from *M. tuberculosis* or *E. coli*. The introduction of P<sub>amr</sub>*dnaG*<sub>Mtb</sub> into the *attB* locus allowed us to remove the native *dnaG* gene without disrupting viability (Fig. 3). Removal of the native copy of *dnaG* from *M. smegmatis* chromosomal DNA was not tolerated following introduction of *E. coli* *dnaG* (P<sub>amr</sub>*dnaG*<sub>Mtb</sub>) into the *attB* site of *M. smegmatis* SCO strain, suggesting that the mycobacterial DnaG have distinct activities or interactions that are essential for replication in these organisms.

Depletion of DnaG only modestly affects the viability of *M. smegmatis*. The depletion of an ideal drug target should result in bacterial cell death or at least in the inhibition of growth. Having confirmed that DnaG is essential for the viability of mycobacteria, even in AEP overproduction backgrounds, we sought to test the effect of controlled DnaG depletion on the viability of *M. smegmatis*. To evaluate DnaG as a potential drug target, we tested whether it is still essential in an AEP-overexpressing background. Genes encoding AEPs were cloned to place them under the control of a chemically inducible promoter (P<sub>amr</sub>) and integrated into the *attB* locus of an *M. smegmatis* SCO mutant carrying both a functional *dnaG* gene and a *dnaG* gene disrupted by the aacC1<sup>R</sup> gene (/H9004 *dnaG*::aacC1<sup>R</sup>). The overexpression of AEPs was confirmed by Western blot analysis (LigD) or quantitative RT-PCR (prim2, prim3, and prim4) (see Fig. S3 in the supplemental material). Next, we selected for mutants lacking an intact copy of *dnaG* (i.e., those carrying Δ*dnaG::aacC1<sup>R</sup>* exclusively). In no case did the overexpression of AEPs rescue the viability defect of *M. smegmatis* lacking an intact chromosomal *dnaG* gene, confirming that *dnaG* is essential and demonstrating that these primases serve nonredundant functions. Next, we tested whether *M. smegmatis* *dnaG* could be replaced by its counterpart from *M. tuberculosis* or *E. coli*. The introduction of P<sub>amr</sub>*dnaG*<sub>Mtb</sub> into the *attB* locus allowed us to remove the native *dnaG* gene without disrupting viability (Fig. 3). Removal of the native copy of *dnaG* from *M. smegmatis* chromosomal DNA was not tolerated following introduction of *E. coli* *dnaG* (P<sub>amr</sub>*dnaG*<sub>Mtb</sub>) into the *attB* site of *M. smegmatis* SCO strain, suggesting that the mycobacterial DnaG have distinct activities or interactions that are essential for replication in these organisms.

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To this end, we grew a conditional DnaG expression mutant carrying only intact dnaGMs (or dnaGMtb) under the control of a chemically inducible Ptet promoter (or Pami), with and without anhydrotetracycline (or acetamide) as an inducer. The growth of the wild-type control strain and conditional mutants was monitored by measuring the optical density at 600 nm (OD600) and determining colony formation; the DnaG protein level was monitored by Western blotting (Fig. 4). Surprisingly, an approximately 83% reduction of DnaG protein levels in M. smegmatis did not (according to the OD600) or only modestly (0.5 to 0.8 log in CFU) affect the growth of bacterial cultures, suggesting that mycobacteria can tolerate substantial depletion of this protein, an observation that has important implications for evaluations of DnaG as a future drug target. The growth inhibition effect was not observed when mineral medium was used to culture wild-type M. smegmatis or conditional mutants (data not shown).

DISCUSSION

The ability to replicate DNA is essential for the viability of every living organism. Consequently, the proteins involved in replication should be essential for viability, at least as long as other proteins cannot complement their functions. Some antibiotics (e.g., doxorubicin, suramin) are known to interfere with the replication machinery of bacteria; however, neither first-line anti-TB drugs nor second-line anti-TB drugs target the mycobacterial replisome (6). We previously evaluated NAD+/H11001-dependent DNA ligase (LigA), which appeared to be essential for mycobacterial viability, as a putative drug target (34). LigA inhibitors that do not affect ATP-dependent ligases were also identified (35, 36). Unexpectedly, depletion of LigA did not significantly affect the growth of mycobacteria (34), largely precluding LigA as a target for new anti-TB drugs. Recently, Biswas et al. developed a colorimetric primase-phosphatase assay as a tool for screening for efficient DnaG inhibitors (32). These researchers used this assay to screen 2,556 small molecules and identified suramin, doxorubicin, and ellagic acid as potential DnaG inhibitors. Here, we evaluated DnaG from mycobacteria as a drug target and engineered an M. smegmatis strain carrying only intact M. tuberculosis dnaG under the control of a chemically inducible promoter. As previously re-
ported (21), dnaG appeared to be essential for the viability of *M. smegmatis*. The genomes of both *M. tuberculosis* and *M. smegmatis* contain multiple copies of a second class of DNA primases belonging to the AEP family. However, we found that DnaG is still essential, even in strains that overexpress these AEP enzymes, establishing that they cannot complement the priming activity of DnaG. The overexpressions of AEPs were confirmed at the protein (LigD) or RNA (Prim2, Prim3, and Prim4) level, and we are not able to exclude the possibility that, in the latter case, some troubles at the translation step occurred. Conversely, the overexpressing genes were originally from the same strain, and common problems (e.g., different GC content, codon usage) for heterogeneous protein expression should not have a place. In contrast to DnaG, individual or even all AEPs were inactivated without affecting the growth of *M. smegmatis* mutants. This observation would suggest that AEPs identified in mycobacteria do not participate in DNA replication and more likely have other roles, including DNA repair.

DnaGs of *M. smegmatis* and *M. tuberculosis* were expressed in *E. coli* and purified to near homogeneity for biochemical studies. The two enzymes were shown to possess primase activity in the presence of Mn$^{2+}$ and ssDNA (24-mer) that was significantly inhibited in the presence of suramin or doxorubicin. However, only doxorubicin appeared to inhibit the growth of wild-type *M. smegmatis* and a Δ*dnag* *M. smegmatis* mutant carrying an intact *dnag* from *M. smegmatis*. The observed resistance to a high concentration of suramin might reflect the limited ability of this compound to permeate mycobacterial cell walls, which are well known for presenting a permeability barrier for hydrophobic and hydrophilic compounds (37, 38). The efficiency of a given inhibitor as an antibacterial drug depends on the intracellular ratio of inhibitor to its target. Thus, the concentration of target protein and the minimal concentration required to reduce cell viability are determinants of the success of treatment. In cases where bacilli are highly sensitive to reductions in the concentration of a given molecule, the molecule in question is a promising drug target. Mycobacterial DnaG may not satisfy this criterion because as little as 17% of wild-type DnaG levels appeared to be sufficient to support the growth of *M. smegmatis*; thus, DnaG may not be a good target for potential anti-TB drugs. As noted above, we observed a similar

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**FIG 3** Complementation of the *M. smegmatis* Δ*dnag* SCO strain. (A) Schematic showing the restriction-digested DNA fragment (1,862 bp) and the size of the internal deletion in the mutated gene (1,180 bp). The *dnag* gene is represented by gray arrows and the internal deletion by white rectangles. The *aacC1* gene (gentamicin resistance cassette) was cloned within the *dnag* gene to facilitate screening of DCO mutants. The *dnag* is essential for the viability of *M. smegmatis*. SCO strains were enriched with intact *dnag* from *M. smegmatis* or *M. tuberculosis* under the control of an inducible promoter (*P_{ami}dnag_{Ms}/dnag_{Ms} or *P_{ami}dnag_{Mt})*. (B) The genotype of selected strains was confirmed by PCR and Southern hybridization analysis.
effect for mycobacterial LigA, another essential replication protein (34). This might suggest that replication proteins are overexpressed in mycobacteria under normal growth conditions, possibly owing to their relatively slow doubling times (39). It has also been reported that 1 to 3% of LigA is sufficient to support E. coli growth under laboratory conditions (40, 41). It is not clear why bacteria express “extra” replication proteins, but it may be related to additional functions performed by these proteins in the cell. The extensive use of LigA or DnaG in DNA damage and repair/recombination pathways might dictate that cells produce a much larger amount of these proteins. The overcapacity in terms of the amount of DnaG available in M. smegmatis suggests that an irreversible inhibitor would be required to eliminate DnaG activity. This likely inhibitory requirement should be taken into account during the screening of new chemicals to target this and related essential replication-associated proteins. The high level of identity between M. smegmatis and M. tuberculosis DnaG and the complementation of M. smegmatis ΔdnaG by intact dnaG in M. tuberculosis would appear to be very useful for the initial testing of DnaG inhibitors identified by random in vitro screening or through rational drug design. Unlike LigA, mycobacterial DnaG was not replaced with its E. coli counterpart. This is consistent with the limited identity between M. smegmatis and E. coli DnaG (32%), which might preclude the interaction of E. coli DnaG with the mycobacterial replisome. The evaluation of DnaG as a putative drug target and construction of M. smegmatis conditional mutants should help in future studies to identify chemicals that efficiently target this essential replication protein in mycobacteria.

FIG 4 Phenotypic analysis of M. smegmatis and the conditionally complemented mutant ΔdnaG. (A) Growth rate analysis of wild-type M. smegmatis and a strain complemented with an intact copy of dnaG, under the control of a tetracycline promoter. Growth rate analyses were performed on rich medium (7H9/oleic acid-albumin-dextrose-catalase [OADC]). OD values are means ± standard errors from three independent experiments. (B) Densitometric analysis of DnaG protein levels in M. smegmatis and ΔdnaG, Western blot analysis with antibodies raised against DnaG of M. smegmatis proteins isolated from cells growing in rich medium in indicated time intervals. Lane 1, M. smegmatis, 0 h; lane 2, ΔdnaG, 0 h; lane 3, M. smegmatis, 6 h; lane 4, ΔdnaG, 6 h; lane 5, ΔdnaG, 12 h; lane 6, ΔdnaG, 12 h; lane 7, M. smegmatis, 24 h; lane 8, ΔdnaG, 24 h.

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