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Evaluation of DNA Primase DnaG as a Potential Target for Antibiotics

Aneta Kuron,a Malgorzata Korycka-Machala,a Anna Brzostek,a Marcin Nowosielski,a Aidan Doherty,b Bozena Dziadek,c Jaroslaw Dziadek6

Institute of Medical Biology, Polish Academy of Sciences, Lodz, Poland6; Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton, United Kingdom6; Department of Immunoparasitology, University of Lodz, Lodz, Poland

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 peroxynitritase 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-
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 nonholomogous 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 (pJet1.2; 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 S′ end of *dnaG* with the upstream region connected to the S′ end of the gene with the downstream region, amplified with the primers shown in Table S2 in the supplemental material. The *aacC1* gene was then introduced between the upstream and downstream regions of *dnaG*. Finally, the P acl screening cassette from pGOAL17 was inserted into the constructs, resulting in the suicide delivery vectors pMK165, pBA148, pMK189, and pMK190 carrying Δ*dnaG*:aacC1′, Δ*prim2, Δprim3*, and Δ*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 pLM2 vector downstream from the Ppromoter (see Table S2). Next, all genes with their Ppromoters were excised from these vectors with HindIII and XbaI and cloned into the integration vector pMV306, generating pMK172, pMK207, pBA145, 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 pKW08Lx vector downstream from the Ppromoter (see Table S2). Next, genes with their Ppromoters were excised from this vector (pMK214) with HindIII and XbaI and cloned into the integration vector pKW08Lx-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, pBA148, 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* (dnaGsm), and *M. tuberculosis* *dnaG* (dnaGmt), cloning into pET1.2, excising with BamHI and HindIII (for dnaGsm), or BamHI/EcoRI (for dnaGmt), 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] 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 Ni^{2+}-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, protein 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 dithiothreitol, 10 mM magnesium acetate (or 10 mM manganese chloride), and 2 μM ssDNA template (5’-tactctcatcgtggaatcctgaca). 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/nmol), the sample 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 −78°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 sensitivity 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 alamarBlue 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 (doxorubicin), filtered (0.22 μm), and used for culture. 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 defined as the lowest drug concentration that prevented a color change. The MICs of doxorubicin (100 μM) and suramin (10 μM), known inhibitors of DnaG (32), which inhibited the activities of *M. smegmatis* 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 liquid 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).

**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/Blast.cgi). In contrast, bacterial primases are distinct from their eukaryotic and archaeal counterparts, 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, respectively). The activity of *M. tuberculosis* DnaG was recently confirmed in an *in vitro* study (32). We used a PT7 Pol-based *E. coli* expression system to overproduce and purify DnaG from *M. smegmatis* and *M. tuberculosis*, and we subsequently used the purified 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 quantifying 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 supplemented with Mn^{2+} or Mg^{2+} and a 24-mer (5’-tactctcatcgtggaatcctgaca) ssDNA template allowed us to confirm the activity of both enzymes (Fig. 1). DnaG activity was suppressed in the presence of doxorubicin (100 μM) and suramin (10 μM), known inhibitors of DnaG (32), which inhibited the activities of *M. smegmatis* 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 liquid 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* mutant 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 doxorubicin, 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 putative AEP-like primase genes. The best characterized of these is PolDom, an AEP that is part of a multidomain enzyme called...
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 is 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 chemically inducible promoter (P<sub>ami</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 (ΔdnaG::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 ΔdnaG::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>ami</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>ami</sub> dnaG<sub>Ec</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. smegma-

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**FIG 1** The purification and activity of M. smegmatis (Ms) and M. tuberculosis (Mt) DnaG proteins. (A) SDS-PAGE analysis. Purified proteins were resolved on a 12% polyacrylamide gel followed by Instant Blue staining. M<sub>n</sub>, molecular weight; nt, nucleotides. (B) Western blot analysis with antibodies raised against DnaG of M. smegmatis. (C) Protein activity assays with Mg<sup>2+</sup>, as described in Materials and Methods.
matis. To this end, we grew a conditional DnaG expression mutant carrying only intact dnaG (or dnaG Mtb) under the control of a chemically inducible P_tet promoter (or P_ami), 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 (OD_{600}) 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 OD_{600}) 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^{+}-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. 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 ΔdnaG *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

**FIG 3** Complementation of the *M. smegmatis* ΔdnaG 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<sub>ami</sub>dnaG<sub>Ms</sub>/dnaG<sub>Mt</sub> or P<sub>tet</sub>dnaG<sub>Ms</sub>). (B) The genotype of selected strains was confirmed by PCR and Southern hybridization analysis.
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, 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, M. smegmatis, 12 h; lane 6, ΔdnaG, 12 h; lane 7, M. smegmatis, 24 h; lane 8, ΔdnaG, 24 h.

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 without a detectable effect on viability or the growth rate suggest that our findings are not limited to nonpathogenic mycobacteria. Thus, a fast-growing nonpathogenic strain expressing DnaG from 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.

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