Evaluation of NAD\(^+\)-Dependent DNA Ligase of Mycobacteria as a Potential Target for Antibiotics\(^\dagger\)

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Mycobacteria contain genes for several DNA ligases, including *ligA*, which encodes a NAD\(^+\)-dependent enzyme that has been postulated to be a target for novel antibacterial compounds. Using a homologous recombination system, direct evidence is presented that wild-type *ligA* cannot be deleted from the chromosome of *Mycobacterium smegmatis*. Deletions of native *ligA* in *M. smegmatis* could be obtained only after the integration of an extra copy of *M. smegmatis* or *Mycobacterium tuberculosis* *ligA* into the attB site of the chromosome, with expression controlled by chemically inducible promoters. The four ATP-dependent DNA ligases encoded by the *M. smegmatis* chromosome were unable to replace the function of LigA. Interestingly, the LigA protein from *M. smegmatis* could be substituted with the NAD\(^+\)-dependent DNA ligase of *Escherichia coli* or the ATP-dependent ligase of bacteriophage T4. The conditional mutant strains allowed the analysis of the effect of LigA depletion on the growth of *M. smegmatis*. The protein level of the conditional mutants was estimated by Western blot analysis using antibodies raised against LigA of *M. tuberculosis*. This revealed that a strong overproduction or depletion of LigA did not affect the growth or survival of mycobacteria under standard laboratory conditions. In conclusion, although NAD\(^+\)-dependent DNA ligase is essential for mycobacterial viability, only low levels of protein are required for growth. These findings suggest that very efficient inhibition of enzyme activity would be required if NAD\(^+\)-dependent DNA ligase is to be useful as an antibiotic target in mycobacteria. The strains developed here will provide useful tools for the evaluation of the efficacy of any appropriate compounds in mycobacteria.

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Tuberculosis (TB) remains a major threat to public health. It is an important infectious disease, causing high morbidity and mortality worldwide, and the situation is made even worse by the emergence of drug-resistant strains of *Mycobacterium tuberculosis* (4). For example, multidrug-resistant (MDR) TB (resistant at least to rifampin and isoniazid) takes longer to treat (up to 2 years) with second-line drugs, which are expensive and have side effects. Even worse is extensively drug-resistant TB, which is resistant to first- and second-line drugs (MDR plus resistance to any fluoroquinolone and at least one of three injectable second-line drugs: capreomycin, kanamycin, or amikacin). Thus, options for treatment are becoming seriously limited, returning TB control to the preantibiotic era (3, 20). Drug resistance in *M. tuberculosis* is not caused by a universal mechanism for all drugs but can be caused by mutations of various chromosomal genes, as identified for MDR occurrence due to the sequential accumulation of mutations in different genes that provide resistance to individual drugs. The mutations connected to resistance can be categorised into targets of current drugs (e.g., *inhA* and *kasA* for isoniazid, *rpoB* for rifampin, and *embCAB* for ethambutol) or enzymes required for the intracellular activation of current drugs (katG for isoniazid, *pncA* for pyrazinamide, and *etaA* for ethionamide) (34). These concerns lead to the conclusion that the identification of novel, sensitive targets or new drugs is necessary for the control of drug-resistant forms of TB.

A requirement for an antibacterial enzyme target is that it be essential for the organism and not present in the host. One such candidate has been proposed to be NAD\(^+\)-dependent DNA ligase (5, 32). DNA ligases are essential constituents of all organisms due to their critical roles in DNA replication and repair. The mechanism of DNA ligation shares common features regardless of the cellular origin of the enzyme, with a key step being the formation of a covalent DNA ligase-adenylate intermediate. Importantly, two classes of DNA ligase that are categorized by whether NAD\(^+\) or ATP is used as the source of adenylate have been identified. While the essential DNA ligases of bacteria are NAD\(^+\) dependent, those used in eukaryotes, archaea, and viruses are ATP dependent. It is this distribution of cofactor specificity that has led to the suggestion that NAD\(^+\)-dependent DNA ligases may be exploited as useful new targets for broad-spectrum antibacterial compounds (5, 24, 29, 32). Indeed, recent studies have begun to make important progress in identifying small molecules that have some specificity towards the inhibition of NAD\(^+\)-dependent DNA ligases (2, 26–28).

Although NAD\(^+\)-dependent DNA ligases appear to be produced in all bacteria, some bacteria encode additional ATP-dependent versions of the proteins (5, 24, 29, 32). This complicates potential strategies to target NAD\(^+\)-dependent DNA ligases with antibiotics, as it is not clear whether the ATP-dependent enzymes would influence the efficacy of any compound. Such factors are particularly relevant to mycobacteria,
because multiple DNA ligases are encoded within their genomes (Fig. 1). Mycobacterial genomes carry a single gene, ligA, that is homologous to NAD$^+$-dependent DNA ligases, with the activity of its expressed product confirmed by in vitro studies (10, 26–28, 33). Three different types of ATP-dependent DNA ligases are encoded in the genomes of mycobacteria, and the biochemical activities of these proteins have also been confirmed (9, 10, 31).

The potential for antibiotics to target NAD$^+$-dependent DNA ligases relies on the fact that these enzymes are believed to be essential for all bacteria due to their participation in DNA replication. However, it is difficult to establish this indispensability in a definitive manner, which is a fundamental requirement if these enzymes are going to be assessed as antibiotic targets. In this report, we undertake a series of experiments that prove directly that ligA is essential in Mycobacterium smegmatis. We demonstrate that this gene can be complemented by non-host NAD$^+$-dependent DNA ligases and the ATP-dependent DNA ligase from bacteriophage T4. A detailed analysis of the amount of DNA ligase in various
strains identifies that the level of protein can vary by around 10-fold, with little effect on growth under standard laboratory conditions. Strains produced during this study will be useful in any detailed evaluation of antibiotics targeting NAD^+ -dependent DNA ligases.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** All strains used in this study were based on M. smegmatis mc^2155 (25) 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). As required, further additions included 0.2% Tween 80 (pH 6.0 to 6.2), 50 g/liter nutrient broth [Difco], 10.0 g/liter glucose). As required, further additions included 0.2% Tween 80 (pH 6.0 to 6.2), 50 g/liter nutrient broth [Difco], 10.0 g/liter glucose).

**Gene cloning strategies.** Standard molecular biology protocols were used for all cloning protocols (22). All PCR products were obtained using thermostable ExTaq polymerase (Takara, Japan), cloned initially into a TA vector (pGEM-T Easy, Promega), and then released by digestion with appropriate restriction enzymes before cloning into the final vectors. To facilitate subcloning into expression vectors, restriction enzyme recognition sites were incorporated into the sequence of the primers. The plasmids used in this work are listed in Table 1.

**Construction of ligA gene replacement vector.** Following strategies reported previously that deleted DNA ligase genes in M. smegmatis (12), a suicidal recombination delivery vector was constructed to perform unmarked deletions in the ligA gene (MSMEG2361) of M. smegmatis. The vector carries the 5’ flanking region of ligA (68 bp) with the upstream region amplified with primers A-GR1 and A-GR2 (Table 2) connected to the 3’ end of the gene (936 bp) and with the downstream region amplified with A-GR3 and A-GR4 primers (Table 2). Note that this cloning results in the 5’ and 3’ fragments of the gene being ligated out of frame, allowing expression of nonfunctional protein only.

**Construction of complementation plasmids.** M. smegmatis genes (ligA, ligB [MSMEG2250], ligC1 [MSMEG6264], ligC2 [MSMEG6265], and ligD [MSMEG5550]), M. tuberculosis ligA (ligA_{Mtb}) (Rv3014), Escherichia coli ligA (ligA_{Ec}) (EC2345), and DNA ligase of bacteriophage T4 (M32518) were amplified by PCR and cloned downstream from the P_start promoter (Tables 1 and 2).

**Testing of ligA essentiality and engineering of conditional mutants.** A two-step recombination protocol (12, 17) was used to select single-crossover (SCO) strains carrying wild-type (wt) and disrupted ligA (ΔligA) at its native locus on the chromosome. Using the strategy outlined in Fig. 2A, the suicidal recombination plasmid DNA (p2NIL/ΔligA) was treated with NaOH (0.2 mM) and integrated into the M. smegmatis mc^2155 chromosome by homologous recombination. The resulting SCO recombinant mutant colonies were blue, Kan^r, and sensitive to any detailed evaluation of antibiotics targeting NAD^+ -dependent DNA ligases.

**TABLE 1. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td>pGemTEasy</td>
<td>T/A cloning</td>
<td>Promega</td>
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<td>p2NIL</td>
<td>Recombination vector, nonreplicating in mycobacteria, Kan^r</td>
<td>17</td>
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<tr>
<td>pGol17</td>
<td>Source of PacI cassette, Amp^r</td>
<td>17</td>
</tr>
<tr>
<td>pJam2</td>
<td>Shuttle vector carrying inducible P_amp promoter, Kan^r</td>
<td>30</td>
</tr>
<tr>
<td>pMV306GM</td>
<td>pMV306 with defective Hyg^r gene (digested with EcoRI, filled-in resultant cohesive ends, and self-ligated) carrying Gm^r gene in KpnI site</td>
<td>This study</td>
</tr>
<tr>
<td>pMVP_amp</td>
<td>pMV306 with P_amp promoter of pJam2 cloned into HindIII-XbaI restriction sites</td>
<td>This study</td>
</tr>
<tr>
<td>pMV306tetR</td>
<td>Mycobacterial integrating vector carrying tetracycline repressor, Km^r</td>
<td>7</td>
</tr>
<tr>
<td>pSE100</td>
<td>Shuttle vector carrying inducible P_tet promoter, Hyg^r</td>
<td>7</td>
</tr>
</tbody>
</table>

**Vectors used for gene replacement**

- **pMK104**: M. smegmatis ΔligA PstI-HindIII fragment including 3’ end and its upstream region (1,754 bp) in p2NIL, Kan^r | This study |
- **pMK106**: M. smegmatis ΔligA HindIII-BamHI fragment including 3’ end and its downstream region (1,741 bp) in pMV306, Kan^r | This study |
- **pMK107**: pMK106 with PacI cassette from pGol17, Kan^r | This study |
- **pMK140**: pMK104 with Hyg^r gene cloned between 5’ and 3’ fragments of ligA (ΔligA::Hyg^r), Kan^r | This study |
- **pMK141**: pMK140 with PacI cassette from pGol17, Kan^r Hyg^r | This study |

**Overproduction vectors**

- **pMK114**: ligA_{Mtb} under P_amp promoter in pJam2, Kan^r | This study |
- **pMK129**: ligA_{Mtb} under P_amp promoter in pJam2, Kan^r | This study |
- **pMK144**: ligC1_{Mtb} under P_amp promoter in pJam2, Kan^r | This study |
- **pMK146**: ligC2_{Mtb} under P_amp promoter in pJam2, Kan^r | This study |
- **pMK113**: ligD_{Mtb} under P_amp promoter in pJam2, Kan^r | This study |
- **pJamligT4**: bacteriophage T4 ligase under P_amp promoter in pJam2, Kan^r | This study |
- **pMK124**: ligA_{Mtb} under P_amp promoter of pMK114 cloned in pMV306, Hyg^r | This study |
- **pMK150**: ligA_{Mtb} under P_amp promoter of pMK129 cloned in pMV306, Hyg^r | This study |
- **pMK155**: ligC1_{Mtb} under P_amp promoter of pMK146 cloned in pMV306GM, Gm^r | This study |
- **pMK152**: ligC2_{Mtb} under P_amp promoter of pMK148 cloned in pMV306GM, Gm^r | This study |
- **pMK154**: ligC2_{Mtb} under P_amp promoter of pMK148 cloned in pMV306GM, Gm^r | This study |
- **pMK150**: ligD_{Mtb} under P_amp promoter of pMK113 cloned in pMV306GM, Gm^r | This study |
- **pMVligA_{Ec}**: ligA_{Ec} cloned under control of P_amp promoter in pMVP_amp, Hyg^r | This study |
- **pMVligT4**: Bacteriophage T4 ligase under P_amp promoter of pJamligT4 cloned in pMV306, Hyg^r | This study |
succeed. Additionally, the suicidal recombination plasmid (p2NILΔligA) was enriched with the HygR gene cloned in a single HindIII site of ΔligA (ΔligA-hyg) and introduced into M. smegmatis. The resulting SCO-2 mutant colonies were blue, KanR, and sensitive to sucrose. The SCO strains were further processed to select for double-crossover (DCO) mutants that were white, KanR, and resistant to sucrose (2%). PCR was used to identify the presence of ΔligA or ΔligD in resultant DCOs.

Further experiments used the strategies outlined in Fig. 2B and Fig. 3 to introduce the complementation plasmids into SCO strains. The resultant strains SCO-ΔligA, SCO-ΔligB, SCO-ΔligAΔligB, SCO-ΔligC, and SCO-ΔligD were selected to be DCO mutant strains in the presence of inducers (0.2% acetamide and 25 μg/ml anhydrotetracycline for FtsZ and Pmar, respectively). If DCOs carrying ΔligA were not identified during the processing of SCO strains with complementing genes, the complemented SCO-2 strains (SCO-2 ΔligAΔligB, SCO-2 ΔligA, and SCO-2 ΔligD) were processed for DCO strains. This approach allowed the selected attempt of DCOs exclusively carrying ΔligA-hyg at the native locus, with the resultant resistance to hygromycin allowing the detection of mutated strains even if they appeared at very low efficiencies.

For all SCO and DCO strains, PCR and Southern hybridization were used to identify the presence of ligA or ΔligA at the native locus on the chromosome. A hybridization probe (to the 3’ end of ligA) was generated by PCR (using primers A-GR3 and MsA-s) (Table 2) and labeled by a nonradioactive primer extension system (DIG-labeling system; Amersham). A-GR4 and MsB-r) (Table 2) and labeled by a nonradioactive primer extension system (DIG-labeling system; Amersham). A-GR4 and MsB-r) (Table 2) and labeled by a nonradioactive primer extension system (DIG-labeling system; Amersham).

**RESULTS**

**NAD⁺-dependent DNA ligase is essential for viability of mycobacteria.** NAD⁺-dependent DNA ligases are highly conserved across all bacterial genomes (32), allowing ready identification of homologous genes by bioinformatics analysis (Fig. 1). The genomes of fast- and slow-growing mycobacteria carry a single gene that is homologous to ligA, with the activity of their expressed NAD⁺-dependent DNA ligases confirmed by in vitro studies (10, 27, 28, 33). Since NAD⁺-dependent DNA ligases are not present in the genomes of cells targeted by mycobacterial pathogens, they have been postulated to be a potential target for new antibiotics (5, 24, 29, 32). The potential usefulness of this approach is dependent on the extent to which these enzymes are essential for the viability of mycobacteria.

To allow a careful evaluation of the essentiality of bacterial ligA, we used M. smegmatis as a model experimental system. A two-step recombination protocol (17) was used to construct SCO strains carrying both wt ligA and a mutated copy of ligA (ΔligA). Further processing of SCO strains for a second step of recombination should generate strains carrying either wt ligA or only the ΔligA gene (mut-DCO) (Fig. 2A). The identification of mut-DCO strains would be possible only if ligA is not essential for the viability of mycobacteria. PCR analysis of more than 50 individual DCO colonies identified wt DCO exclusively, confirming that knockout mutations in ligA are lethal for mycobacteria.

Even though the wt gene is essential, we wondered if it
would be possible to delete it from the bacterial chromosome if another copy of the gene at a different location supported the expression of required protein. Moreover, by controlling expression from the additional gene by an inducible promoter, a conditional mutant might be obtained. To prepare such mutant strains, \( M.\) \textit{smegmatis} \( \text{ligA} \) (\( \text{ligAMs} \)) and \( M.\) \textit{tuberculosis} \( \text{ligA} \) (\( \text{ligAMt} \)) were cloned under the control of the tetracycline (\( \text{P}_{\text{tet}} \)) or acetamidase (\( \text{P}_{\text{ami}} \)) promoter. These recombinant genes were introduced into SCO strains by integration into the host chromosome (genes induced from \( \text{P}_{\text{ami}} \)) or as a self-replicating vector (genes induced from \( \text{P}_{\text{tet}} \)). The resultant mutant strains were processed by PCR and Southern hybridization analysis, indicating that DCO strains were both \( \text{wt} \) (\( \text{ligA} \)) and mutant type (\( \Delta\text{ligA} \)). Numbers above the lanes of each gel represent the following samples: 1, 1-kb DNA ladder; 2, \( M.\) \textit{smegmatis} \( \text{wt} \) control; 3, \( \text{p2NIL}\text{ ligA} \) plasmid; 4, SCO mutant strains. DCO mutant strains are indicated in the figure, with those containing \( \Delta\text{ligA} \) highlighted by an asterisk (*). Note that the middle PstI recognition site (A) was present within the deleted region of \( \text{ligA} \), thus causing the \( \Delta\text{ligA} \) band detected by Southern hybridization to be larger than the \( \text{wt} \text{ ligA} \) band. The Southern hybridization probe was amplified using the \( 3' \) undeleted end of \( \text{ligA} \).

![FIG. 2. NAD\(^+\)-dependent \( \text{ligA} \) is essential for viability of \( M.\) \textit{smegmatis}. (A) A deleted version of \( \text{ligA} \) (\( \Delta\text{ligA} \)) of \( M.\) \textit{smegmatis} was constructed by PCR and introduced into the suicide recombination vector \( \text{p2NIL} \). SCO mutants carrying both \( \text{ligA} \) and \( \Delta\text{ligA} \) were obtained by the integration of plasmid DNA (\( \text{p2NIL}\Delta\text{ligA} \)) into regions of the chromosome that flank the gene of interest. SCO mutant strains were processed directly for DCO mutant strains. The genotype of selected strains (\( >50 \)) was confirmed by PCR, indicating that all DCO strains carried \( \text{wt} \text{ ligA} \) exclusively. (B) SCO strains from A were enriched with intact \( \text{ligA} \) from \( M.\) \textit{smegmatis} or \( M.\) \textit{tuberculosis} controlled by an inducible promoter (\( \text{P}_{\text{ami}} \text{ ligAMs}/\text{ligAMt} \)). The genotype of selected strains was confirmed by PCR and Southern hybridization analysis, indicating that DCO strains were both \( \text{wt} \) (\( \text{ligA} \)) and mutant type (\( \Delta\text{ligA} \)). Numbers above the lanes of each gel represent the following samples: 1, 1-kb DNA ladder; 2, \( M.\) \textit{smegmatis} \( \text{wt} \) control; 3, \( \text{p2NIL}\Delta\text{ligA} \) plasmid; 4, SCO mutant strains. DCO mutant strains are indicated in the figure, with those containing \( \Delta\text{ligA} \) highlighted by an asterisk (*). Note that the middle PstI recognition site (A) was present within the deleted region of \( \text{ligA} \), thus causing the \( \Delta\text{ligA} \) band detected by Southern hybridization to be larger than the \( \text{wt} \text{ ligA} \) band. The Southern hybridization probe was amplified using the \( 3' \) undeleted end of \( \text{ligA} \).}

\( \text{NAD}^+\)-dependent DNA ligase cannot be replaced by over-production of ATP-dependent DNA ligases of \textit{mycobacteria}. In addition to an \( \text{NAD}^+\)-dependent DNA ligase, mycobacteria encode three different ATP-dependent ligases (9, 10, 31). In fact, \( M.\) \textit{smegmatis} has the potential to express four ATP-dependent DNA ligases since it has two closely related copies of \( \text{ligC} \) (Fig. 1). BLAST analysis identified that each of these ATP-dependent DNA ligases contains a domain that is well conserved in other DNA ligases, as indicated by their Pfam nomenclature (8): Pfam01068 relates to the conserved catalytic (adenylation) domain of DNA ligases. Notably, this domain is present in the ATP-dependent DNA ligase of \textit{enterobacterio-}
phage T4 (Fig. 1), which is able to replace the function of ligAEc (13). Although the essential nature of ligA shows that the ATP-dependent DNA ligases cannot normally replace LigA, we wondered if these proteins may be able to replace the function of LigA if they were overexpressed. Such a phenomenon could lead to the selection of resistant strains if LigA were to be inactivated with an antibiotic drug. Therefore, by following the procedures outlined above to obtain conditional mutants, we assessed the potential redundancy of function between the mycobacterial DNA ligases by testing whether ligA is still essential in genetic backgrounds that overexpress ATP-dependent ligases.

FIG. 3. NAD⁺-dependent ligA of M. smegmatis can be substituted with NAD⁺-dependent ligA of E. coli or ATP-dependent ligase of enterobacteriophage T4. SCO strains carrying both ligA and ΔligA (Fig. 2) were enriched with ligAEc (A) or ligT4 (B) controlled by a Pami promoter. DCO mutant strains resulting from the processing of these SCO strains were both wt (ligA) and mutant type (ΔligA). The genotype of selected DCO strains was confirmed by PCR (A and B) and Southern hybridization (C) analyses. Numbers above the lanes of each gel represent the following samples: 1, 1-kb DNA ladder; 2, M. smegmatis wt control; 3, p2NILΔligA plasmid; 4, SCO mutant strains; 5, DCO mutant strains carrying wt ligA; 6, DCO mutant strains carrying ΔligA. In the Southern hybridization shown in C, 6A and 6B represent the complementation by ligAEc and ligT4, respectively. Note that the middle PstI recognition site (A) was present within the deleted region of ligA (ΔligA), thus causing the ΔligA band detected by Southern hybridization to be larger than the wt ligA band. The Southern hybridization probe was amplified using the 3' undeleted end of ligA.

The Southern hybridization probe was amplified using the 3' undeleted end of ligA.
To simplify the DCO screening and to allow the examination of a larger number of colonies, the SCO strain was modified by the introduction of a hygromycin resistance cassette into ΔligA (ΔligA::Hygr). This allowed the resultant mut-DCO and wt DCO to be distinguished by supplementation of hygromycin B into the screening media, with only the mut-DCO strains being able to grow under such conditions. The ATP-dependent ligases of *M. smegmatis* (ligB, ligC1, ligC2, and ligD) were cloned under the control of the *P*<sub>ami</sub> promoter and introduced into the *attB* site of an SCO strain. Although we did not have antibodies to test of the level of expression for each of these proteins, the expression system is known to be efficient (6), and it clearly worked for the *ligA* homologs (Fig. 2A). The screening for DCO strains was performed in the presence of the *P*<sub>ami</sub> inducer and in the presence or absence of hygromycin B. No colonies with the expected DCO phenotype (white and Km<sup>r</sup>) grew in the presence of hygromycin B in the media. For SCO strains carrying a gene for each of the ATP-dependent ligases, all DCO strains obtained without hygromycin revealed the wt genotype (wt DCO) when verified by PCR (data not shown). These results demonstrate that ATP-dependent DNA ligases cannot substitute for NAD<sup>+</sup>-dependent enzymes, again confirming the essentiality of *ligA* in *M. smegmatis*.

*E. coli* NAD<sup>+</sup>-dependent DNA ligase and ATP-dependent ligase of bacteriophage T4 can substitute for LigA in mycobacteria. The data described above show that the NAD<sup>+</sup>-dependent DNA LigA of *M. smegmatis* can be substituted with its ortholog from *M. tuberculosis*. This result is expected since there is very close homology across mycobacterial gene sequences. Since mycobacterial genomes are G+C rich, we wondered if they may have evolved specialized versions of *ligA*. We tested whether complementation of LigA<sub>Mm</sub> might extend to gene sequences that are less well conserved. In these experiments, we used genes that are much less G+C rich, namely, those that are the paradigms for DNA ligases that participate in replication: the NAD<sup>+</sup>-dependent DNA LigA from the gram-negative bacterium *E. coli*; the ATP-dependent ligase from enterobacteriophage T4. Both genes were amplified by PCR, cloned under the control of the *P*<sub>ami</sub> promoter, and introduced into the *attB* site of a SCO strain already carrying ΔligA. The resultant strains carrying wt *ligA*, Δ*ligA*, and either *ligA<sub>E</sub>* or the ATP-dependent ligase of bacteriophage T4 (*ligT4*) expressed from the *P*<sub>ami</sub> promoter were processed to obtain DCO strains. PCR and Southern hybridization analysis revealed mut-DCO strains without an intact copy of *ligA<sub>Mm</sub>* (Fig. 3). Thus, *ligA<sub>Mm</sub>* could be complemented with either *ligA<sub>E</sub>* or *ligT4*. Significantly, in the latter case, the resultant strains of *M. smegmatis* carry genes for ATP-dependent DNA ligases exclusively.

The replacement of LigA<sub>Mm</sub> with its counterpart from *M. tuberculosis* or *E. coli* did not produce a significant effect on the growth of *M. smegmatis* (Fig. 4A and B). Mutant strains carrying *ligT4* to complement *ligA* from *M. smegmatis* grew more slowly, taking an extra 24 h to reach stationary phase, especially if its expression was not induced to a high level with acetylame (*P*<sub>ami</sub> inducer) (Fig. 4C). The growth dynamics of the strain carrying *ligT4* were most affected by a delay in achieving exponential growth. Since each culture was inoculated with the same number of actively growing cells, such a delay in growth is likely to result from an increase in doubling time compared to the wt strain. Although the specific reasons behind this delay are not yet known, it is clear that *M. smegmatis* is sensitive to the type of replicative DNA ligase that is present within the cell.

**The growth of *M. smegmatis* is insensitive to the level of LigA.** A reliable antibiotic target should be essential for bacterial viability, and a significant decrease in its activity should have a similarly significant effect on bacterial growth. To assess this property of LigA<sub>Mm</sub>, the constructed conditional mutant strains carrying a single *ligA* gene under the control of inducible promoters (*P*<sub>ect</sub>*P*<sub>ami</sub>) were used to analyze the influence of the LigA amount on bacterial growth.

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**FIG. 4.** *M. smegmatis* carrying only ATP-dependent ligases is defective in growth. The *M. smegmatis* wt control strain (wt) and conditional mutant strains carrying ligase genes driven by *P*<sub>ami</sub> promoter were grown in Middlebrook 7H9/oleic acid-albumin-dextrose catalase medium with and without the induction of *P*<sub>ami</sub>. All conditional mutant strains carried a single intact copy of the named *ligA* substituting for a deletion in *M. smegmatis* *ligA* as follows: (A) Δ*ligA-P<sub>ami</sub>* Δ*ligA<sub>Mm</sub>*; (B) Δ*ligA-P<sub>ami</sub>* Δ*ligA<sub>E</sub>*; (C) Δ*ligA-P<sub>ami</sub>* Δ*ligT4*; Δ*ligA* mutant complemented with the gene for T4 DNA ligase. The growth of each culture was monitored by optical density analysis. Growth experiments were repeated three times, with the representative result being presented in the figure. OD<sub>600</sub>, optical density at 600 nm.
Strains were grown as required, and the level of LigA was estimated using polyclonal antibodies raised against a recombinant form of LigA_09. Initial experiments confirmed that this antibody had cross-reactivity with native LigA expressed from the chromosome of _M. smegmatis_ (data not shown). The mutant strains were able to grow without inducer supplementing the media, indicating that both promoters are weak and allow low levels of constitutive expression. The protein analysis showed that the P_ami promoter leaks more than P_ani but its fully induced level of expression was much higher (Fig. 5). Nevertheless, these observations are likely to be due to the fact that _P_ami expressed genes from plasmid DNA (five to six copies) but that _P_ani expressed genes from a single copy in the chromosome. The strains expressing different levels of LigA protein were cultured: the wt _M. smegmatis_ strain, the ΔligA-P_ani ligA strain growing without acetamide in the medium, and the ΔligA-P_am ligA strain growing in medium supplemented with anhydrotetracycline. The growth of these three strains was determined by optical density measurement, and the level of LigA was determined by Western blot analysis (Fig. 5). The Western blot analysis revealed a large overproduction of LigA in the ΔligA-P_ani ligA strain growing in the presence of inducer, up to 11 times more than that of the wt strain as detected by densitometry (data not shown). By contrast, very low amounts of LigA were present in the ΔligA-P_ami ligA mutant strain (about three times less than the wt strain). However, the growth dynamics of the strains were not affected by the different levels of LigA and were comparable to the growth of the wt strain (Fig. 5).

It is likely that drug treatments decrease the level of functional target protein, which inhibits or prevents bacterial growth over long-term exposure. To determine if the depletion of LigA over long periods may affect the viability of _M. smegmatis_, the wt strain and ΔligA-P_ani ligA mutant strains were incubated for 3 weeks. No inducer was added to the medium in this experiment, so there was limited expression of LigA in the ΔligA-P_ani ligA strains. The number of viable cells was counted, and the level of LigA was determined by Western blot analysis. During these experiments, lasting for 21 days, no significant differences in viability were observed between a wt strain carrying normal levels of LigA and mutant strains carrying as little as 30% of LigA (data not shown). Thus, the results presented here suggest that any compound targeting LigA would need to reduce the activity of this essential enzyme by more than 70% if it is to be able to act effectively as an antibiotic.

**DISCUSSION**

Mycobacteria contain genes for several ATP-dependent DNA ligases and a single NAD^+-dependent DNA ligase encoded by _ligA_. Previous studies are consistent with _ligA_ being essential in _M. smegmatis_ (9) and _M. tuberculosis_ (23). The data presented here support these suggestions and provide further direct evidence that NAD^+-dependent DNA ligases are essential in bacteria. Thus, these experiments in _mycobacteria_ support proposals from experiments with other bacteria, including _E. coli_ (14), _Salmonella enterica_ serovar Typhimurium (18), _Bacillus subtilis_ (19), and _Staphylococcus aureus_ (11). These findings confirm that NAD^+-dependent DNA ligases may provide useful targets for broad-spectrum antibacterial compounds (5, 24, 29, 32) and suggest that it is worthwhile to focus effort on identifying specific inhibitors of them (2, 26–28).

Complementation of _M. smegmatis_ ΔligA strains by expression of _ligA_ from _E. coli_ confirms that the function of this protein is conserved across organisms that are widely divergent in evolutionary terms. By contrast, the mycobacterial ATP-dependent DNA ligases are unable to replace the function of LigA. This is due simply to the use of ATP as the cofactor since the _M. smegmatis_ ΔligA strains are complemented by the ATP-dependent DNA ligase of bacteriophage T4. In terms of the protein sequences of different DNA ligases, the catalytic region is well conserved among all ATP-dependent DNA ligases used in this study (Fig. 1). Since the _M. smegmatis_ ATP-dependent DNA ligases were unable to produce the same complementation as the T4 DNA ligase, this shows that some other parts of their polypeptide sequences influence their activity and prevent them from functioning in DNA replication. This observation is consistent with findings that the ATP-de-
dependent DNA ligases of bacteria participate in DNA repair pathways rather than DNA replication (1, 24, 29).

The observation that the M. smegmatis ΔligA strains can be complemented by the ATP-dependent DNA ligase of bacteriophage T4 is consistent with findings for other bacteria (13, 18, 21). Although bacteriophage T4 does not naturally infect mycobacteria, this observation raises the possibility that if genes encoding ATP-dependent ligases were transferred to the mycobacterial chromosome (e.g., by horizontal gene transfer), they may be able to substitute for LigA and therefore impact the targeting of DNA ligases by antibiotics. On the other hand, horizontal transmission of resistance factors is not generally seen with tubercle bacilli.

Evaluation of the expression of LigA shows that M. smegmatis grows similarly across levels of expression of the protein that varied by approximately 10-fold. This is reminiscent of findings for E. coli, where it has been estimated that 1 to 3% of LigA is sufficient to support growth under laboratory conditions (14, 16). It is not fully understood why bacterial cells have evolved to express such an additional “capacity” of DNA ligase as a standard, but it may relate to the fact that these enzymes participate in many aspects of DNA metabolism. The activity of ligase required for standard laboratory conditions may be relatively low, since the enzyme will be involved primarily with DNA replication. However, at certain times, such as after extensive DNA damage, the increased flux through DNA repair/recombination pathways will mean that the cells require a much larger DNA ligase activity. The cells may therefore find it an advantage to have a built-in extra capacity so that they can respond quickly to times of increased DNA stress. Future experiments will test the effects of DNA damage on the strains produced in this study.

The high level of identity between M. smegmatis and M. tuberculosis DNA ligases and the complementation of the M. smegmatis ΔligA mutant strain with intact ligAMt, without a detectable effect on viability or growth rate suggest that our findings are not limited to nonpathogenic mycobacteria. Our experiments with complemented strains of M. smegmatis raise at least two important issues that should be evaluated in the context of targeting NAD⁺-dependent DNA ligases with antibiotics. First, complementation with DNA ligase from bacteriophage T4 raises the possibility that proteins other than mycobacterial LigA may influence the efficacy of an inhibitor. Second, the overcapacity in terms of the amount of LigA available to M. smegmatis suggests that an irreversible inhibitor will need to shut down LigA activity extremely efficiently. Presently, it is unclear if inhibitors of NAD⁺-dependent DNA ligases would be able to act so specifically and efficiently. Although good progress is being made in the development of potential compounds to target NAD⁺-dependent DNA ligases (2, 26–28), the concerns raised in this study make it clear that these compounds will need to be tested in good experimental models. The mycobacterial strains described here will provide useful tools for such a detailed evaluation.

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