Evaluation of NAD+-Dependent DNA Ligase of Mycobacteria as a Potential Target for Antibiotics[▽]

Malgorzata Korycka-Machala,[1] Ewelina Rychta,[2] Anna Brzostek,[1] Heather R. Sayer,[3]
Anna Rumijowska-Galewicz,[1] Richard P. Bowater,[3] and Jarosław Dziadek[1]*

*Centre of Medical Biology, Polish Academy of Sciences, Lodz, Poland[1]; Department of Genetics of Microorganisms, University of
Lodz, Lodz, Poland[2]; and School of Biological Sciences, University of East Anglia, Norwich, United Kingdom[3]*

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.

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 appear in 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,

* Corresponding author. Mailing address: Centre of Medical Biology, Polish Academy of Sciences, Lodowa 106, 93-231 Lodz, Poland. Phone: 48 42 2723610. Fax: 48 42 2723630. E-mail: jdziadek@cbm.pan.pl.
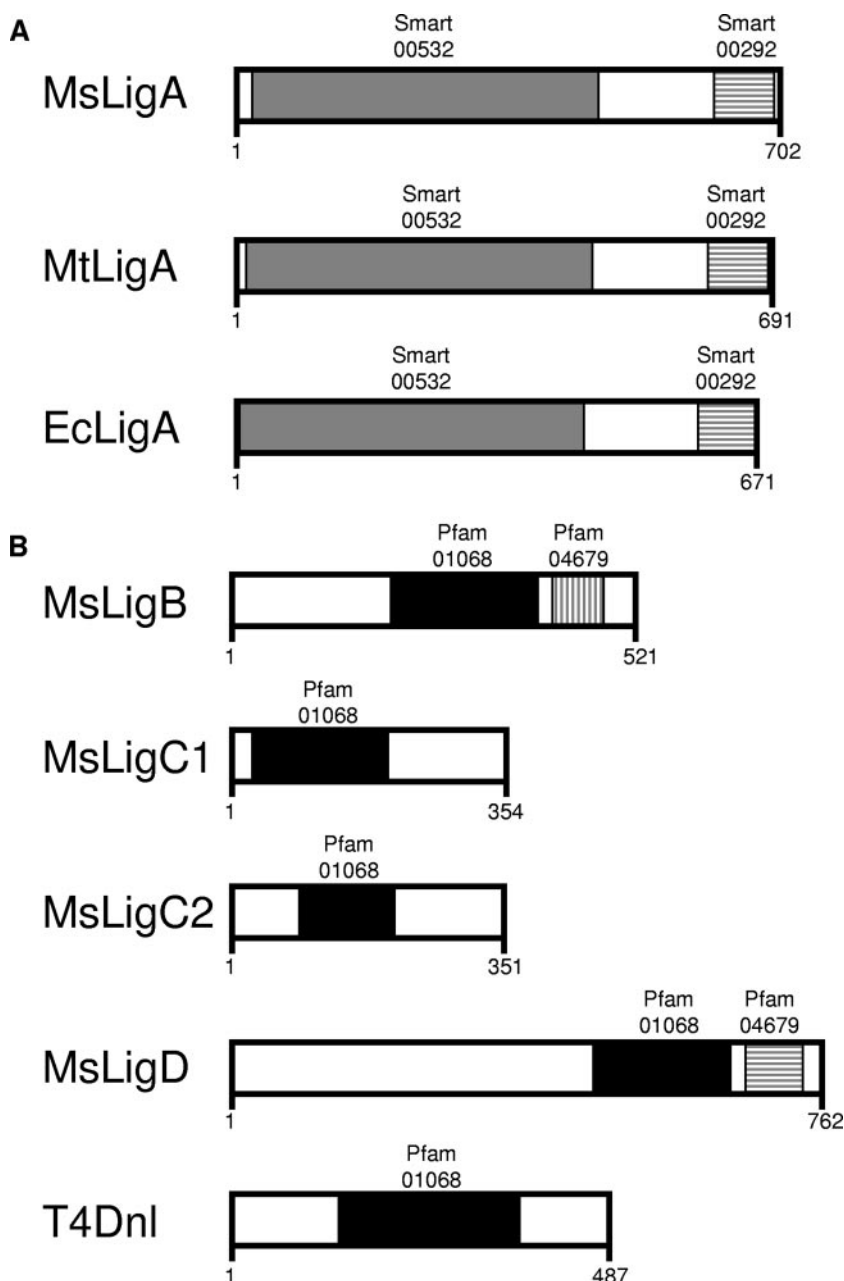
FIG. 1. DNA ligases of mycobacteria, *E. coli*, and bacteriophage T4. A schematic diagram of conserved domains within the various DNA ligases used in this study is shown. The number of amino acids in each specific protein is indicated. Approximate relative positions of domains conserved within more than one of the proteins are shown by the shaded regions. Domains were identified from their reference within the Pfam (8) or SMART (15) database, as indicated. (A) NAD$^+$-dependent ligases from *M. smegmatis* (MsLigA), *M. tuberculosis* (MtLigA), and *E. coli* (EcLigA). (B) ATP-dependent DNA ligases from *M. smegmatis* (MsLigB, MsLigC1, MsLigC2, and MsLigD) and bacteriophage T4 (T4Dnl).

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

TABLE 1. Plasmids used in this study

Plasmid	Description	Source or reference
Cloning vectors		
pGemTEasy	T/A cloning	Promega
pMV306	Mycobacterial integrating vector, Hyg^r	Med-Immune Inc.
p2NIL	Recombination vector, nonreplicating in mycobacteria, Kan^r	17
pGoal17	Source of *Pac*I cassette, Amp^r	17
pJam2	Shuttle vector carrying inducible P$_{ami}$ promoter, Kan^r	30
PMV306GM	pMV306 with defective Hyg^r gene (digested with EcoRI, filled-in resultant cohesive ends, and self-ligated) carrying Gm^r gene in KpnI site	This study
pMVP$_{ami}$	pMV306 with P$_{ami}$ promoter of pJam2 cloned into HindIII-XbaI restriction sites	This study
pMV306tetR	Mycobacterial integrating vector carrying tetracycline repressor, Km^r	7
pSE100	Shuttle vector carrying inducible P$_{tet}$ promoter, Hyg^r	7
Vectors used for gene replacement		
pMK104	*M. smegmatis* 5′ ΔligA PstI-HindIII fragment including 5′ 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 pMK104, Kan^r	This study
pMK107	pMK106 with *Pac*I cassette from pGoal17, Kan^r	This study
pMK140	pMK104 with Hyg^r gene cloned between 5′ and 3′ fragments of *ligA* (ΔligA::Hyg^r), Kan^r Hyg^r	This study
pMK141	pMK140 with *Pac*I cassette from pGoal17, Kan^r Hyg^r	This study
Overproduction vectors		
pMK114	*ligA*$_{Ms}$ under P$_{ami}$ promoter in pJam2, Kan^r	This study
pMK129	*ligA*$_{Ms}$ under P$_{ami}$ promoter in pJam2, Kan^r	This study
pMKB144	*ligB*$_{Ms}$ under P$_{ami}$ promoter in pJam2, Kan^r	This study
pMKC146	*ligC1*$_{Ms}$ under P$_{ami}$ promoter in pJam2, Kan^r	This study
pMKC148	*ligC2*$_{Ms}$ under P$_{ami}$ promoter in pJam2, Kan^r	This study
pMK113	*ligD*$_{Ms}$ under P$_{ami}$ promoter in pJam2, Kan^r	This study
pJamligT4	bacteriophage T4 ligase under P$_{ami}$ promoter in pJam2, Kan^r	This study
pMK124	*ligA*$_{Ms}$ under P$_{ami}$ promoter of pMK114 cloned in pMV306, Hyg^r	This study
pMK130	*ligA*$_{Mt}$ under P$_{ami}$ promoter of pMK129 cloned in pMV306, Hyg^r	This study
pMK155	*ligB*$_{Ms}$ under P$_{ami}$ promoter of pMKB144 cloned in pMV306GM, Gm^r	This study
pMK152	*ligC1*$_{Ms}$ under P$_{ami}$ promoter of pMKC146 cloned in pMV306GM, Gm^r	This study
pMK154	*ligC2*$_{Ms}$ under P$_{ami}$ promoter of pMKC148 cloned in pMV306GM, Gm^r	This study
pMK150	*ligD*$_{Ms}$ under P$_{ami}$ promoter of pMK113 cloned in pMV306GM, Gm^r	This study
pMVligA$_{Ec}$	*ligA*$_{Ec}$ cloned under control of P$_{ami}$ promoter in pMVP$_{ami}$, Hyg^r	This study
pMVligT4	Bacteriophage T4 ligase under P$_{ami}$ promoter of pJamligT4 cloned in pMV306, Hyg^r	This study

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^{2}155 (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 μg/ml hygromycin, 7.5 μg/ml gentamicin, and 25 μg/ml kanamycin. Mycobacterial transformants were selected on Middlebrook 7H10 agar plates enriched with albumin-dextrose-sodium chloride containing kanamycin (25 μg/ml), gentamicin (7.5 μg/ml), or hygromycin (50 μg/ml).

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 re-

combination delivery vector was constructed to perform unmarked deletions in the *ligA* gene (MSMEG2361) of *M. smegmatis*. The vector carried the 5′ end 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* [MSMEG2280], *ligC1* [MSMEG6264], *ligC2* [MSMEG6263], and *ligD* [MSMEG5550]), *M. tuberculosis ligA* (*ligA*$_{Mt}$) (Rv3014), *Escherichia coli ligA* (*ligA*$_{Ec}$) (EC2345), and DNA ligase of bacteriophage T4 (M32518) were amplified by PCR and cloned downstream from the P$_{ami}$ promoter (Tables 1 and 2). Mycobacterial ligases were cloned first into the BamHI-XbaI sites of pJam2, and the cloned genes and P$_{ami}$ promoter were then excised with HindIII and XbaI and cloned into the integration vector pMV306$_{HygR}$ or pMV306$_{GmR}$. *ligA*$_{Ec}$ was introduced into the single XbaI site of pMV306-P$_{ami}$ under the control of the acetamidase promoter. The gene for T4 DNA ligase was introduced into the BamHI site of pJam2, excised from this vector with HindIII, and cloned into pMV306. Finally, *ligA* of *M. smegmatis* was cloned into pSE100 downstream from the P$_{tet}$ promoter (Tables 1 and 2).

Testing of *ligA* essentiality and engineering of conditional mutants. A two-step recombination protocol (12, 17) was used to obtain 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 (p2NILΔligA) was treated with NaOH (0.2 mM) and integrated into the *M. smegmatis* mc^{2}155 chromosome by homologous recombination. The resulting SCO recombinant mutant colonies were blue, Kan^r, and sensitive to

TABLE 2. Primer sequences used for PCR amplification of gene sequences

Amplified region	Primer	Sequence	Product size (bp)
Primers used to amplify DNA for targeted gene replacement			
ligA 5′ flanking region, sense	A-GR1	5′-<u>CTGCAG</u>TCTCGCGCGTCGCGCGCTCG-3′	1,754
ligA 5′ flanking region, reverse	A-GR2	5′-<u>AAGCTT</u>CGCCGCTCGTCGGCGTCAGG-3′	
ligA 3′ flanking region, sense	A-GR3	5′-<u>AAGCTT</u>GGCGACACCGTGGTGATCCGC-3′	1,741
ligA 3′ flanking region, reverse	A-GR4	5′-<u>GGATCC</u>AGCCGAGACCACAGGGCGGG-3′	
Primers used to clone genes for complementation experiments			
ligA$_{Ms}$ gene, sense	MsA-s	5′-<u>GGATCC</u>GTGAGCGAGAAGGCAACCG-3′	2,106
ligA$_{Ms}$ gene, reverse	MsA-r	5′-<u>TCTAGA</u>CTACTCGGCCGGCTCGACC-3′	
ligB$_{Ms}$ gene, sense	MsB-s	5′-<u>AGATCT</u>ATGGATGTCCGACCCGCG-3′	1,566
ligB$_{Ms}$ gene, reverse	MsB-r	5′-<u>TCTAGA</u>TCAGTCGCGCTCGTAGAAC3′	
ligC1$_{Ms}$ gene, sense	MsC1-s	5′-<u>GGATCC</u>ATGGACCTGCCCGTGCTGC-3′	1,065
ligC1$_{Ms}$ gene, reverse	MsC1-r	5′-<u>TCTAGA</u>CTAAACCCGGCACGATGTC-3′	
ligC2$_{Ms}$ gene, sense	MsC2-s	5′-<u>AGATCT</u>ATGGGAAGGATGGACTTGC-3′	1,056
ligC2$_{Ms}$ gene, reverse	MsC2-r	5′-<u>TCTAGA</u>TCACTGTTCCTCCAGCACG-3′	
ligD$_{Ms}$ gene, sense	MsD-s	5′-<u>GGATCC</u>GTGGCGAGGCATCCTTGGG-3′	2,289
ligD$_{Ms}$ gene, reverse	MsD-r	5′-<u>TCTAGA</u>CTATTCCCACACAACCTC-3′	
ligA$_{Mt}$ gene, sense	MtbA-s	5′-<u>AGATCT</u>GTGAGCTCCCCAGACGCCG-3′	2,076
ligA$_{Mt}$ gene, reverse	MtbA-r	5′-<u>TCTAGA</u>TTACGTTCGTGACGCGGG-3′	
ligA$_{Ec}$ gene, sense	EcA-s	5′-<u>TCTAGA</u>ATGGAATCAATCGAACAAC-3′	2,016
ligA$_{Ec}$ gene, reverse	EcA-r	5′-<u>TCTAGA</u>TTAGCTACCCAGCAAACGC-3′	
T4Dnl gene, sense	T4-s	5′-<u>GGATCC</u>ATGATTCTTAAAATTCTGAACG-3′	1,464
T4Dnl gene, reverse	T4-r	5′-<u>GGATCC</u>TCATAGACCAGTTACCTCATG-3′	

sucrose. Additionally, the suicidal recombination plasmid (p2NILΔ*ligA*) was enriched with the Hyg^r gene cloned in a single HindIII site of Δ*ligA* (Δ*ligA*::*hyg*) and introduced into *M. smegmatis*. The resulting SCO-2 mutant colonies were blue, Kan^r, Hyg^r, and sensitive to sucrose. The SCO strains were further processed to select for double-crossover (DCO) mutants that were white, Kan^s, and resistant to sucrose (2%). PCR was used to identify the presence of *ligA* or Δ*ligA* 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-P$_{ami}$ *ligA$_{Ms}$*, SCO-P$_{tet}$ *ligA$_{Ms}$*, SCO-P$_{ami}$ *ligA$_{Mt}$*, SCO-P$_{ami}$ *ligA$_{Ec}$*, SCO-P$_{ami}$ *ligB*, SCO-P$_{ami}$ *ligC1*, SCO-P$_{ami}$ *ligC2*, SCO-P$_{ami}$ *ligD*, and SCO-P$_{ami}$ T4Dnl were processed to select for DCO mutant strains in the presence of inducers (0.2% acetamide and 25 ng/ml anhydrotetracycline for P$_{ami}$ and P$_{tet}$, respectively). If DCOs carrying Δ*ligA* were not identified during the processing of SCO strains with complementing genes, the complemented SCO-2 strains (SCO-2 P$_{ami}$ *ligB*, SCO-2 P$_{ami}$ *ligC1*, SCO-2 P$_{ami}$ *ligC2*, and SCO-2 P$_{ami}$ *ligD*) were processed for DCO strains. This approach allowed the attempted selection of DCOs exclusively carrying Δ*ligA*::*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 Δ*ligA* 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-r) (Table 2) and labeled by a nonradioactive primer extension system (DIG-labeling system; Amersham).

Preparation of antibodies against LigA$_{Mt}$. Cloning and purification of a recombinant form of LigA$_{Mt}$ has been described previously (33). Note that this protein contains a His$_{10}$ tag within an extra 21 amino acids (2.5 kDa) at the N terminus. LigA$_{Mt}$ was purified using nickel affinity chromatography (His-Bind column; Novagen) and was used to raise a primary rabbit polyclonal antibody (Davids Biotechnologie).

Total protein isolation and Western blotting. Preparation of mycobacterial cell lysates by bead beating (using 0.1-mm zirconia beads) and detection of LigA by Western blotting using polyclonal antibodies were done essentially as described previously (6). The total protein concentration was determined by the Bradford method (22). For quantitative immunoblotting, the same concentration of total protein extracts was loaded onto sodium dodecyl sulfate-polyacrylamide gels. Western blots were processed using the Amersham Pharmacia ECL chemiluminescence kit and protocol. The LigA bands were visualized on Hyperfilm

ECL (Amersham) and scanned for densitometry with FluorChem (Alpha Innotech Corp.).

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* (Δ*ligA*). Further processing of SCO strains for a second step of recombination should generate strains carrying either wt *ligA* or only the Δ*ligA* 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.

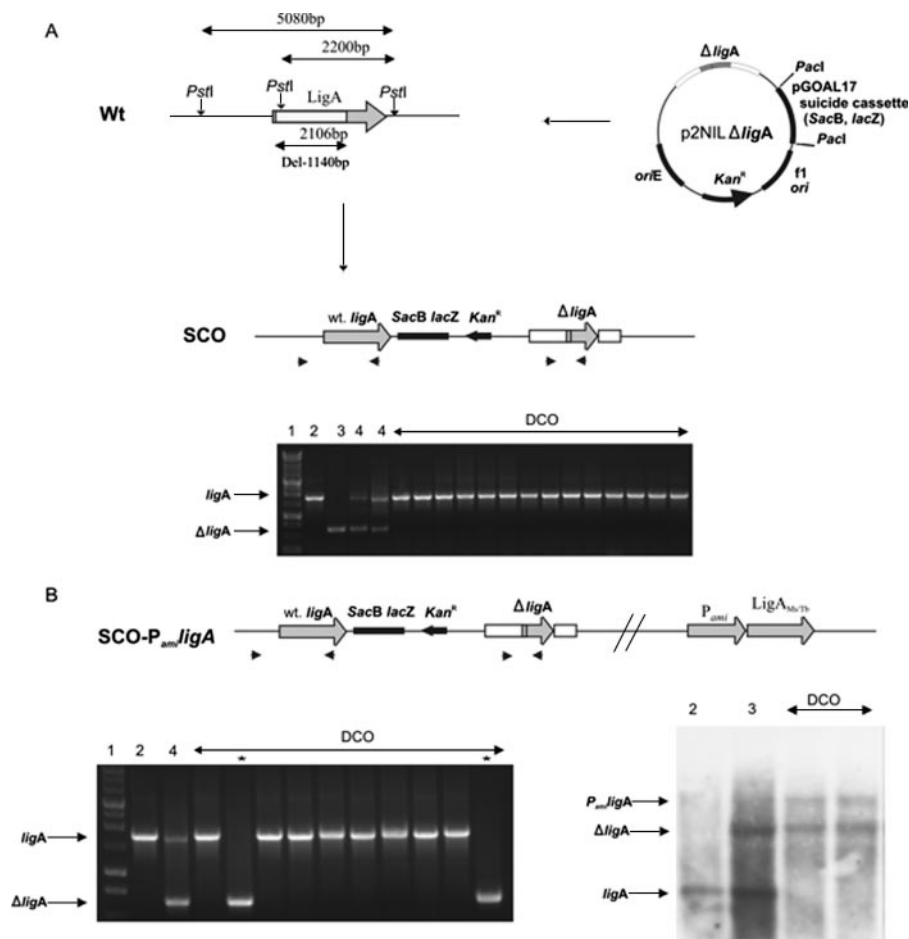Even though the wt gene is essential, we wondered if it

FIG. 2. NAD$^+$-dependent *ligA* is essential for viability of *M. smegmatis*. (A) A deleted version of *ligA* (Δ*ligA*) of *M. smegmatis* was constructed by PCR and introduced into the suicide recombination vector p2NIL. SCO mutants carrying both *ligA* and Δ*ligA* were obtained by the integration of plasmid DNA (p2NILΔ*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 wt *ligA* exclusively. (B) SCO strains from A were enriched with intact *ligA* from *M. smegmatis* or *M. tuberculosis* controlled by an inducible promoter (P$_{ami}$ *ligA$_{Ms}$*/*ligA$_{Mt}$*). The genotype of selected strains was confirmed by PCR and Southern hybridization analysis, indicating that DCO strains were both wt (*ligA*) and mutant type (Δ*ligA*). Numbers above the lanes of each gel represent the following samples: 1, 1-kb DNA ladder; 2, *M. smegmatis* wt control; 3, p2NILΔ*ligA* plasmid; 4, SCO mutant strains. DCO mutant strains are indicated in the figure, with those containing Δ*ligA* highlighted by an asterisk (*). Note that the middle PstI recognition site (A) was present within the deleted region of *ligA* (Δ*ligA*), thus causing the Δ*ligA* 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*.

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. smegmatis ligA* (*ligA$_{Ms}$*) and *M. tuberculosis ligA* (*ligA$_{Mt}$*) were cloned under the control of the tetracycline (P$_{tet}$) or acetamidase (P$_{ami}$) promoter. These recombinant genes were introduced into SCO strains by integration into the host chromosome (genes induced from P$_{ami}$) or as a self-replicating vector (genes induced from P$_{tet}$). The resultant mutant strains (SCO-P$_{tet}$/P$_{ami}$ *ligA$_{Ms}$*/*ligA$_{Mt}$*) carrying wt *ligA*, Δ*ligA*, and P$_{tet}$/ P$_{ami}$ *ligA$_{Ms}$*/*ligA$_{Mt}$* were processed in the presence of the appropriate inducer to obtain DCO mutant strains. mut-DCO strains carrying *ligA$_{Ms}$*/*ligA$_{Mt}$* controlled by the P$_{ami}$ or P$_{tet}$ promoter as the sole intact copy of *ligA* were identified by PCR

and Southern hybridization (Fig. 2B). The successful engineering of such strains confirmed the essentiality of *ligA* in mycobacteria and showed that *ligA$_{Mt}$* can replace its *M. smegmatis* ortholog.

NAD$^+$-dependent DNA ligase cannot be replaced by overproduction of ATP-dependent DNA ligases of mycobacteria. In addition to an NAD$^+$-dependent DNA ligase, mycobacteria encode three different ATP-dependent ligases (9, 10, 31). In fact, *M. smegmatis* has the potential to express four ATP-dependent DNA ligases since it has two closely related copies of *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 enterobacterio-
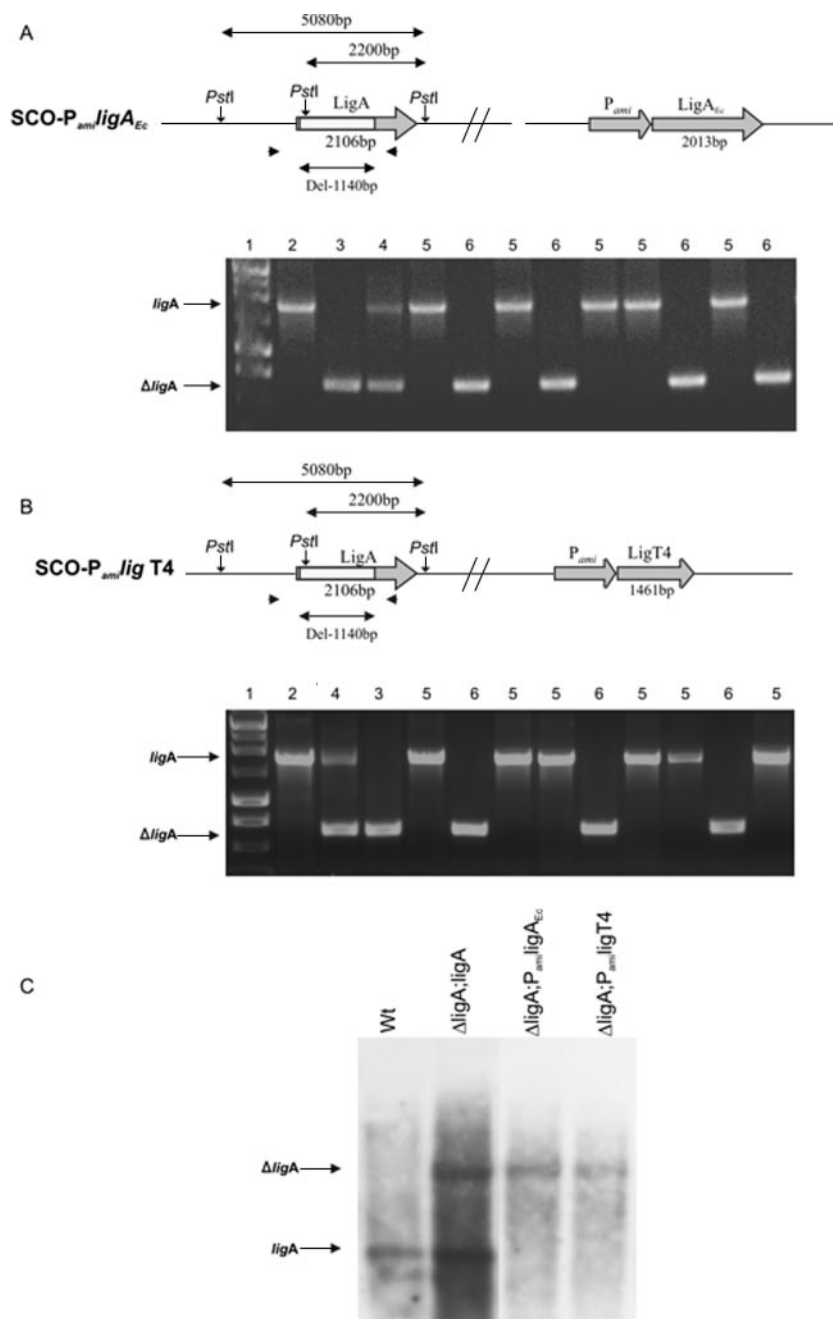
FIG. 3. NAD$^+$-dependent *ligA* of *M. smegmatis* can be substituted with NAD$^+$-dependent *ligA* of *E. coli* or ATP-dependent ligase of enterobacteriophage T4. SCO strains carrying both *ligA* and Δ*ligA* (Fig. 2) were enriched with *ligA*$_{Ec}$ (A) or *lig*T4 (B) controlled by a P$_{ami}$ promoter. DCO mutant strains resulting from the processing of these SCO strains were both wt (*ligA*) and mutant type (Δ*ligA*). 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, p2NILΔ*ligA* plasmid; 4, SCO mutant strains; 5, DCO mutant strains carrying wt *ligA*; 6, DCO mutant strains carrying Δ*ligA*. In the Southern hybridization shown in C, 6A and 6B represent the complementation by *ligA*$_{Ec}$ and *lig*T4, respectively. Note that the middle PstI recognition site (A) was present within the deleted region of *ligA* (Δ*ligA*), thus causing the Δ*ligA* 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*.

phage T4 (Fig. 1), which is able to replace the function of *ligA*$_{Ec}$ (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.

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 Δ*ligA* (Δ*ligA*::Hyg^r). 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*ami* 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*ami* inducer and in the presence or absence of hygromycin B. No colonies with the expected DCO phenotype (white and Km^s) 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^+-dependent enzymes, again confirming the essentiality of *ligA* in *M. smegmatis*.

E. coli NAD^+-dependent DNA ligase and ATP-dependent ligase of bacteriophage T4 can substitute for LigA in mycobacteria. The data described above show that the NAD^+-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*Ms* 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^+-dependent DNA LigA from the gram-negative bacterium *E. coli* and the ATP-dependent ligase from enterobacteriophage T4. Both genes were amplified by PCR, cloned under the control of the P*ami* promoter, and introduced into the *attB* site of an SCO strain already carrying Δ*ligA*. The resultant strains carrying wt *ligA*, Δ*ligA*, and either *ligA*_Ec_ or the ATP-dependent ligase of bacteriophage T4 (*lig*T4) expressed from the P*ami* promoter were processed to obtain DCO strains. PCR and Southern hybridization analysis revealed mut-DCO strains without an intact copy of *ligA*_Ms_ (Fig. 3). Thus, *ligA*_Ms_ could be complemented with either *ligA*_Ec_ 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_Ms_ 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 *lig*T4 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 acetamide (P*ami* inducer) (Fig. 4C). The growth dynamics of the strain carrying *lig*T4 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
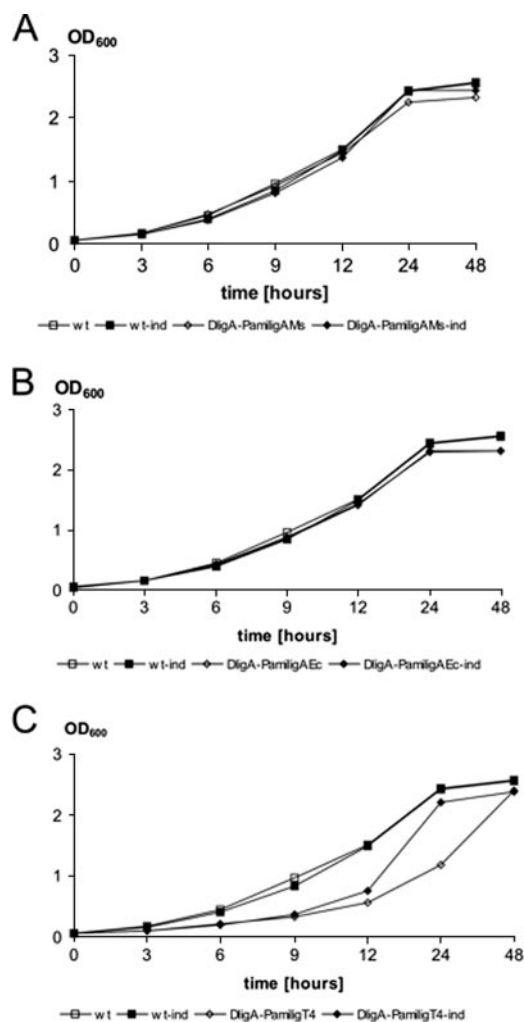

FIG. 4. *M. smegmatis* carrying only ATP-dependent ligases is defective in growth. The *M. smegmatis* wt control strain (wt) and conditional mutant strains carrying ligase genes driven by P*ami* promoter were grown in Middlebrook 7H9/oleic acid-albumin-dextrose catalase medium with and without the induction of P*ami*. 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*ami* *ligA*_Ms_, Δ*ligA* mutant complemented with *M. smegmatis ligA*; (B) Δ*ligA*-P*ami* *ligA*_Ec_, Δ*ligA* mutant complemented with *E. coli ligA*; (C) Δ*ligA*-P*ami* *lig*T4, 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_600_, optical density at 600 nm.

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_Ms_, the constructed conditional mutant strains carrying a single *ligA* gene under the control of inducible promoters (P*tet*/P*ami*) were used to analyze the influence of the LigA amount on bacterial growth.
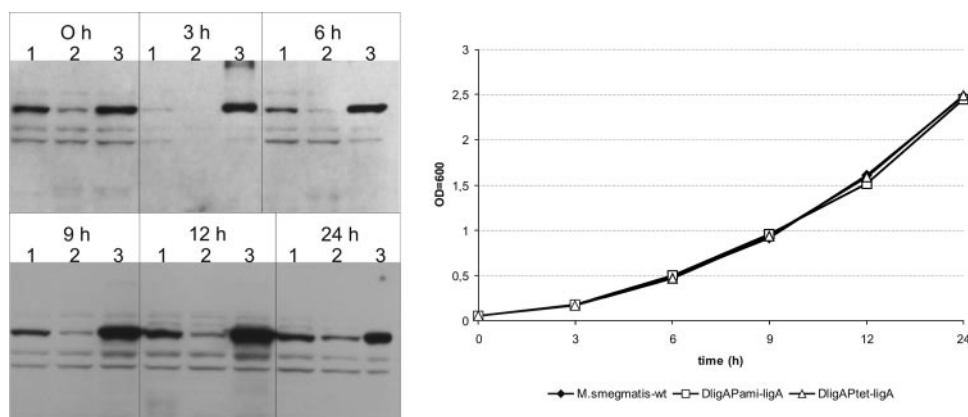
FIG. 5. Growth of *M. smegmatis* is insensitive to the amount of expressed LigA. As shown in the panel on the right, optical density (OD) analysis determined the extent of growth of *M. smegmatis* strains carrying a natural level of LigA (lane 1, wt strain), a low level of LigA (lane 2, Δ*ligA*-P$_{ami}$ *ligA$_{Ms}$*, without inducer), and an overproduced level of LigA (lane 3, Δ*ligA*-P$_{tet}$ *ligA$_{Ms}$*, with inducer). In the left panel, the level of LigA was determined by Western blot analysis with antibodies raised against LigA of *M. tuberculosis*. For each lane, 12 μg of total proteins was loaded.

Strains were grown as required, and the level of LigA was estimated using polyclonal antibodies raised against a recombinant form of LigA$_{Ms}$. 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 leaky and allow low levels of constitutive expression. The protein analysis showed that the P$_{tet}$ promoter leaks more than P$_{ami}$, but its fully induced level of expression was much higher (Fig. 5). These observations are likely to be due to the fact that P$_{tet}$ expressed genes from plasmid DNA (five to six copies) but that P$_{ami}$ 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 Δ*ligA*-P$_{ami}$ *ligA* strain growing without acetamide in the medium, and the Δ*ligA*-P$_{tet}$ *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 Δ*ligA*-P$_{tet}$ *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 Δ*ligA*-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 Δ*ligA*-P$_{ami}$ *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 Δ*ligA*-P$_{ami}$ *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 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 the most direct genetic evidence yet obtained 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* Δ*ligA* 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 not due simply to the use of ATP as the cofactor since the *M. smegmatis* Δ*ligA* 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-

pendent DNA ligases of bacteria participate in DNA repair pathways rather than DNA replication (1, 24, 29).

The observation that the *M. smegmatis* Δ*ligA* 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* Δ*ligA* mutant strain with intact *ligA*$_{Mt}$ 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.

ACKNOWLEDGMENTS

REFERENCES

1. **Bowater, R. P., and A. J. Doherty.** 2006. Making ends meet: repairing breaks in bacterial DNA by non-homologous end-joining. PLoS Genet. **2:**93–99.
2. **Brotz-Oesterhelt, H., I. Knezevic, S. Bartel, T. Lampe, U. Warnecke-Eberz, K. Ziegelbauer, D. Habich, and H. Labischinski.** 2003. Specific and potent inhibition of NAD+-dependent DNA ligase by pyridochromanones. J. Biol. Chem. **278:**39435–39442.
3. **Centers for Disease Control and Prevention.** 2006. Emergence of Mycobacterium tuberculosis with extensive resistance to second-line drugs—worldwide, 2000–2004. Morb. Mortal. Wkly. Rep. **55:**301–305.
4. **Dahle, U. R., P. Sandven, E. Heldal, and D. A. Caugant.** 2003. Continued low rates of transmission of *Mycobacterium tuberculosis* in Norway. J. Clin. Microbiol. **41:**2968–2973.
5. **Doherty, A. J., and S. W. Suh.** 2000. Structural and mechanistic conservation in DNA ligases. Nucleic Acids Res. **28:**4051–4058.
6. **Dziadek, J., M. V. Madiraju, S. A. Rutherford, M. A. Atkinson, and M. Rajagopalan.** 2002. Physiological consequences associated with overproduction of Mycobacterium tuberculosis FtsZ in mycobacterial hosts. Microbiology **148:**961–971.
7. **Ehrt, S., X. V. Guo, C. M. Hickey, M. Ryou, M. Monteleone, L. W. Riley, and D. Schnappinger.** 2005. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res. **33:**e21.
8. **Finn, R. D., J. Mistry, B. Schuster-Bockler, S. Griffiths-Jones, V. Hollich, T. Lassmann, S. Moxon, M. Marshall, A. Khanna, R. Durbin, S. R. Eddy, E. L. L. Sonnhammer, and A. Bateman.** 2006. Pfam: clans, web tools and services. Nucleic Acids Res. **34:**D247–D251.
9. **Gong, C., P. Bongiorno, A. Martins, N. C. Stephanou, H. Zhu, S. Shuman, and M. S. Glickman.** 2005. Mechanism of nonhomologous end-joining in mycobacteria: a low-fidelity repair system driven by Ku, ligase D and ligase C. Nat. Struct. Mol. Biol. **12:**304–312.
10. **Gong, C., A. Martins, P. Bongiorno, M. Glickman, and S. Shuman.** 2004. Biochemical and genetic analysis of the four DNA ligases of mycobacteria. J. Biol. Chem. **279:**20594–20606.
11. **Kaczmarek, F. S., R. P. Zaniewski, T. D. Gootz, D. E. Danley, M. N. Mansour, M. Griffor, A. V. Kamath, M. Cronan, J. Mueller, D. Sun, P. K. Martin, B. Benton, L. McDowell, D. Biek, and M. B. Schmid.** 2001. Cloning and functional characterization of an NAD$^+$-dependent DNA ligase from *Staphylococcus aureus*. J. Bacteriol. **183:**3016–3024.
12. **Korycka-Machala, M., A. Brzostek, S. Rozalska, A. Rumijowska-Galewicz, R. Dziedzic, R. Bowater, and J. Dziadek.** 2006. Distinct DNA repair pathways involving RecA and nonhomologous end joining in Mycobacterium smegmatis. FEMS Microbiol. Lett. **258:**83–91.
13. **Lavesa-Curto, M., H. Sayer, D. Bullard, A. MacDonald, A. Wilkinson, A. Smith, L. Bowater, A. Hemmings, and R. Bowater.** 2004. Characterisation of a temperature-sensitive DNA ligase from *Escherichia coli*. Microbiology **150:**4171–4180.
14. **Lehman, I. R.** 1974. DNA ligase: structure, mechanism, and function. Science **186:**790–797.
15. **Letunic, I., R. R. Copley, B. Pils, S. Pinkert, J. Schultz, and P. Bork.** 2006. SMART 5: domains in the context of genomes and networks. Nucleic Acids Res. **34:**D257–D260.
16. **Modrich, P., and I. R. Lehman.** 1973. Deoxyribonucleic acid ligase. A steady state kinetic analysis of the reaction catalyzed by the enzyme from Escherichia coli. J. Biol. Chem. **248:**7502–7511.
17. **Parish, T., and N. G. Stoker.** 2000. Use of a flexible cassette method to generate a double unmarked Mycobacterium tuberculosis tlyA plcABC mutant by gene replacement. Microbiology **146:**1969–1975.
18. **Park, U. E., B. M. Olivera, K. T. Hughes, J. R. Roth, and D. R. Hillyard.** 1989. DNA ligase and the pyridine nucleotide cycle in *Salmonella typhimurium*. J. Bacteriol. **171:**2173–2180.
19. **Petit, M. A., and S. D. Ehrlich.** 2000. The NAD-dependent ligase encoded by yerG is an essential gene of Bacillus subtilis. Nucleic Acids Res. **28:**4642–4648.
20. **Raviglione, M.** 2006. XDR-TB: entering the post-antibiotic era? Int. J. Tuberc. Lung Dis. **10:**1185–1187.
21. **Ren, Z. J., R. G. Baumann, and L. W. Black.** 1997. Cloning of linear DNAs in vivo by overexpressed T4 DNA ligase: construction of a T4 phage hoc gene display vector. Gene **195:**303–311.
22. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol. Microbiol. **48:**77–84.
24. **Shuman, S., and C. D. Lima.** 2004. The polynucleotide ligase and RNA capping enzyme superfamily of covalent nucleotidyltransferases. Curr. Opinion. Struct. Biol. **14:**757–764.
25. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.**

1990. Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis. Mol. Microbiol. **4:**1911–1919.

26. **Srivastava, S. K., D. Dube, V. Kukshal, A. K. Jha, K. Hajela, and R. Ramachandran.** 7 June 2007, posting date. NAD+-dependent DNA ligase (Rv3014c) from Mycobacterium tuberculosis: novel structure-function relationship and identification of a specific inhibitor. Proteins doi:10.1002/prot.21457.

27. **Srivastava, S. K., D. Dube, N. Tewari, N. Dwivedi, R. P. Tripathi, and R. Ramachandran.** 2005. Mycobacterium tuberculosis NAD+-dependent DNA ligase is selectively inhibited by glycosylamines compared with human DNA ligase I. Nucleic Acids Res. **33:**7090–7101.

28. **Srivastava, S. K., R. P. Tripathi, and R. Ramachandran.** 2005. NAD+-dependent DNA ligase (Rv3014c) from Mycobacterium tuberculosis. Crystal structure of the adenylation domain and identification of novel inhibitors. J. Biol. Chem. **280:**30273–30281.

29. **Tomkinson, A. E., S. Vijayakumar, J. M. Pascal, and T. Ellenberger.** 2006. DNA ligases: structure, reaction mechanism, and function. Chem. Rev. **106:**687–699.

30. **Triccas, J. A., T. Parish, W. J. Britton, and B. Gicquel.** 1998. An inducible expression system permitting the efficient purification of a recombinant antigen from Mycobacterium smegmatis. FEMS Microbiol. Lett. **167:**151–156.

31. **Weller, G. R., B. Kysela, R. Roy, L. Tonkin, E. Scanlan, M. Della, S. K. Devine, J. P. Day, A. Wilkinson, F. d'Adda di Fagagna, K. Devine, R. P. Bowater, P. Jeggo, S. P. Jackson, and A. J. Doherty.** 2002. Identification of a DNA non-homologous end-joining complex in bacteria. Science **297:**1686–1689.

32. **Wilkinson, A., J. Day, and R. Bowater.** 2001. Bacterial DNA ligases. Mol. Microbiol. **40:**1241–1248.

33. **Wilkinson, A., H. Sayer, D. Bullard, A. Smith, J. Day, T. Kieser, and R. Bowater.** 2003. NAD+-dependent DNA ligases of *Mycobacterium tuberculosis* and *Streptomyces coelicolor*. Proteins **51:**321–326.

34. **Zhang, Y., C. Vilcheze, and W. R. Jacobs, Jr.** 2005. Mechanisms of drug resistance in *Mycobacterium tuberculosis*, p. 115–142. *In* S. T. Cole, K. D. Eisenach, D. N. McMurray, and W. R. Jacobs, Jr. (ed.), Tuberculosis and the tubercle bacillus. ASM Press, Washington, DC.