Tuberculosis, caused by *Mycobacterium tuberculosis*, causes more deaths than almost any other infectious agent. It is estimated that one-third of the world population is latently infected with *M. tuberculosis* (38), with most individuals carrying the organism without showing disease symptoms. During infection, *M. tuberculosis* bacteria reside within host macrophages, where they are likely to encounter reactive oxygen and nitrogen species (19, 34) which can damage macromolecules, including DNA. Therefore, the ability to repair damaged DNA and to maintain chromosomal integrity will play a role in the persistence of *M. tuberculosis* in host tissues, and presumably in the maintenance of a reservoir of future infection. Indeed, *M. tuberculosis* strains carrying mutations in genes involved in DNA repair have been shown to be attenuated for infection in a nonhuman primate model of infection (8), and an *M. tuberculosis* strain deficient in nucleotide excision repair (NER) shows reduced pathogenicity in mice (5). NER is a method of DNA repair by which a single-stranded oligonucleotide surrounding a base carrying a bulky adduct can be removed, which allows DNA polymerase to fill in the gap created, thereby replacing the damaged DNA.

In *Escherichia coli*, in addition to its role in NER, UvrD appears to play roles in recombination (17), replication (9, 14, 18), plasmid rolling-circle replication (2), and the mismatch repair pathway (16), an alternative method of DNA repair commonly found in prokaryotes but absent in *M. tuberculosis* (36). Thus, UvrD homologues appear to play roles in a number of distinct pathways, and many of these roles appear to involve the ability to turn over protein-DNA complexes (11), which may be independent of helicase activity (1, 15).

*M. tuberculosis* and other mycobacterial species possess two homologues of UvrD, annotated UvrD1 and UvrD2 (3). Previously, we have shown that UvrD1 of *M. tuberculosis* is a DNA helicase with 3’-5’ polarity and with an unwinding preference for nicked DNA resembling an NER intermediate and for substrates resembling stalled replication forks (4). Furthermore, an *M. tuberculosis* uvrD1 mutant strain exhibited increased sensitivity to DNA damaging agents commonly processed by NER (J. Houghton, C. Güthlein, B. Springer, E. C. Böttger, and E. O. Davis, unpublished data), similar to a *Mycobacterium smegmatis* uvrD1 mutant strain (10). Thus, it would appear that it is UvrD1 that functions in NER in *M. tuberculosis*. Interestingly, *M. tuberculosis* UvrD1 also suppresses DNA strand-exchange reactions catalyzed by RecA (28), and *M. smegmatis* UvrD1 appears to play a role in recombining (10), supporting that mycobacterial UvrD1 has further roles outside NER.

UvrD2 has an unusual protein domain structure, with an N-terminal UvrD domain (Pfam00580) typical of superfAMILY I helicases linked to a C-terminal HRDC domain (Pfam00570) typical of superfAMILY II helicases, with an intervening domain carrying a tetracysteine motif (31). In the case of *Mycobacterium smegmatis* UvrD2, the C-terminal HRDC domain is not required for *in vitro* DNA helicase activity (31), while the tetracysteine domain (but not the tetracysteine motif) is required for DNA unwinding. Surprisingly, it was found that Ku, a DNA-binding protein which plays a role in the nonhomologous end-joining (NHEJ) pathway, could restore helicase ac-
tivity to the truncated protein lacking the tetracysteine and HRDC domains. The unusual domain structure of UvrD2 suggests that it may have a distinct biological function from that of UvrD1, which is more similar to typical UvrD-like helicases.

In contrast to uvrD1, uvrD2 has been shown to be essential in *M. smegmatis* (31). Both uvrD1 and uvrD2 have been predicted to be essential in *M. tuberculosis* by a global transposon mutagenesis approach (27); nevertheless, we have been able to isolate a mutant strain in which uvrD1 is inactivated (our unpublished data). In this study, we demonstrate directly that uvrD2 is essential in *M. tuberculosis* and investigate which properties of the encoded protein are required for its essential function.

**MATERIALS AND METHODS**

**Bacterial strains and media.** The strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH5α (Invitrogen) was used for all plasmid construction and site-directed mutagenesis (25). A streptomycin-resistant mutant strain of *M. smegmatis* DH5α (Invitrogen) was used for all plasmid construction and site-directed mutagenesis. A streptomycin-resistant mutant strain in which uvrD2 has been shown to be essential in *M. tuberculosis* by a global transposon mutagenesis approach (27); nevertheless, we have been able to isolate a mutant strain in which uvrD1 is inactivated (our unpublished data). In this study, we demonstrate directly that uvrD2 is essential in *M. tuberculosis* and investigate which properties of the encoded protein are required for its essential function.

**ATPase assay.** The ability of *M. tuberculosis* UvrD2 to hydrolyze ATP was measured by linking ATP hydrolysis to NADH oxidation and measuring it spectrophotometrically as described previously (4), with the modification of using 10 to 500 μM UvrD2. Briefly, reaction mixtures (200 μM) were incubated at 37°C for 15 min in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 10% glycerol in the presence of 0.05 to 10 μM single-stranded DNA (ssDNA) (23 nucleotides [nt]), 2 mM ATP, 0.2 mM NADH, and 2 mM phosphoenolpyruvate, along with 4.7 U lactate dehydrogenase and 5.3 U pyruvate kinase (Sigma-Aldrich). NADH concentrations were measured using a bovine serum albumin (BSA) protein assay kit (Thermo Scientific) according to the manufacturer’s instructions.

**Helicase assay.** A fluorometric assay was used to observe unwinding of a partial duplex DNA substrate. An oligonucleotide complex was designed to include 22 complementary nucleotides with a 3’-ROX and 3’-BHQ-2 groups. In a duplex, the fluorescence of the ROX dye is quenched by the BHQ-2 moiety. Upon unwinding of the duplex DNA, the separation of the fluorophore from the quencher increases fluorescence. Labeled oligonucleotides were mixed at a 1:1.5 molar ratio (fluorophore to quencher) to minimize free ROX, heated to 90°C, and allowed to cool to room temperature.
temperature for 2 h. Reaction mixtures (30 μl) contained 100 nM DNA substrate and 500 nM UvD2 in helicase buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 10% glycerol). To prevent reannealing of the substrate, a 500 nM capture oligonucleotide corresponding to the 22 bp of the fluorophore oligonucleotide complementary to the quencher strand was added. The reaction mix was equilibrated to 37°C, and the reaction was initiated by addition of 5 mM ATP. DNA unwinding was monitored by measuring ROX fluorescence on a Gemini XPS microplate spectrofluorometer (Molecular Devices), with excitation at 584 nm and emission measured at 612 nm. Reported initial unwinding rates are average for at least 4 independent experiments.

Streptavidin displacement assay. In order to measure DNA translocation, the ability of helicase proteins to displace proteins from DNA was used (23). A radiolabeled oligonucleotide was incubated with 30 nM streptavidin for 15 min to allow the streptavidin to bind to the DNA. Free biotin (6 μM) was added to act as a trap for displaced and nonannealed streptavidin, and reactions in 10-μl mixtures were started by the addition of 200 nM UvD2 or a mutant derivative. The reaction mixtures were incubated for 15 min at 37°C, and the reactions were quenched by the addition of 10 μl stop solution (1 M NaCl, 200 mM EDTA, pH 8.0, 40% glycerol, 0.3% bromophenol blue) containing 50 nM nonbiotinylated oligonucleotide to bind to the protein and prevent band shifting. Control reactions lacking protein were performed and showed no dissociation of streptavidin from the biotinylated oligonucleotide. The reaction products were analyzed by electrophoresis through a 10-cm 15% polyacrylamide gel in 89 mM Tris-borate, 2 mM EDTA. The gels were dried under vacuum and exposed to a phosphorimager screen. Radiolabeled bands were visualized using a Storm860 phosphorimager and processed using ImageQuant software (Molecular Dynamics).

**Plasmid construction.** A targeting construct for \( M. tuberculosis \) UvD2 (Rv3198c) was constructed by amplifying the flanking genomic regions by PCR. The 5′ region was amplified from \( M. tuberculosis \) genomic DNA by a PCR using primers 7f and 7r (Table 2), incorporating NdeI and BglII sites, respectively, and the 3′ region was amplified with primers 8f and 8r, introducing BglII and NdeI sites, respectively. The PCR products were cloned sequentially into the suicide plasmid pAW26, using a Stratagene QuikChange XL kit and the oligonucleotides indicated in Table 2. The resulting plasmids, pAW26 (24) to generate plasmid pAW24, pKPI86, and pKPI86 E508A, was used. PCR products spanning the uvd2 promoter and full-length gene and truncated copies covering residues 1 to 600 and 1 to 640 were cloned into pKPI86 to give plasmids pAW26, pAW27, and pAW28, respectively. To introduce point mutations, site-directed mutagenesis was carried out on plasmid pAW26, using a Stratagene QuikChange XL kit, and the oligonucleotides indicated in Table 2. The resulting plasmids, pAW29 [uvD2(E508A)], were confirmed to show that the point mutations had been introduced and that no other bases had been changed in the gene sequence.

**Genetic procedures.** Plasmids were introduced into \( M. tuberculosis \) by electroporation as described previously (37). Single-crossover transformants (SCOs) were selected on plates containing hygromycin. Double-crossover transformants (DCOs) were isolated by streaking cells onto plates lacking antibiotics, followed by selection on medium containing streptomycin. Colonies were screened for hygromycin sensitivity and then by PCR using primers D2delchkF and D2delchkR (Table 2). We created a merodiplast strain by electroporating the uvd2 SCO with pAW24 and selecting for kanamycin/hygromycin resistance. As a control, the empty vector pKPI86 was electroporated into the pKPI86 SCO strain. DCOs were isolated and screened as described above. DCOs were confirmed by Southern analysis using an ECL direct nucleic acid labeling system (Amersham), using a PCR probe located in the 5′-flanking region of the deletion. Complement switching was carried out by transforming the complemented uvd2 deletion strain with plasmids pAW26, pAW27, pAW28, pAW29, and pAW30. Colonies were selected for gentamicin resistance and then screened for kanamycin sensitivity. A PCR-based screen was then used to confirm gene replacement, using an internal uvd2 primer (uvD2int3) and vector-specific primers (pKPI86R and pKPI803R).

**RESULTS**

UvD2 is essential in \( M. tuberculosis \). Initially, we were interested in assessing the role of UvD2 in DNA damage repair in \( M. tuberculosis \), particularly in relation to whether there is an overlap in function between UvD1 and UvD2. Although both uvd1 and uvd2 had been predicted to be essential (27), we had been able to isolate a mutant in uvd1, so we attempted to remove uvd2 by allelic exchange.

After transformation of the targeting plasmid into \( M. tuberculosis \) 1424, Hyg³ colonies were checked by PCR and Southern

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**TABLE 2. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2delchkF</td>
<td>GCATGACACCACGCACTTC</td>
</tr>
<tr>
<td>D2delchkR</td>
<td>CAGGATGCTGGCCAAATAGC</td>
</tr>
<tr>
<td>Q266R_F</td>
<td>CGTGGGGCGAGGGACATGCTTTCG</td>
</tr>
<tr>
<td>Q266R_R</td>
<td>GGAGAATGCTACGGGAACTTGCTGTCCAG</td>
</tr>
<tr>
<td>E508A_F</td>
<td>GCATGGGCACCTAGGAGATCTCACGTACCCGTGGATCCTCTAGAGT</td>
</tr>
<tr>
<td>E508A_R</td>
<td>TGGACCGGTGTGCGTGCTGG</td>
</tr>
<tr>
<td>D2outhF</td>
<td>TGAGAACCGTGTTGCTGTCCAG</td>
</tr>
<tr>
<td>D2outhR</td>
<td>ACTTGTCGTCAGCACGTCG</td>
</tr>
<tr>
<td>UvD2int3</td>
<td>TGCTGTCGGAGTCAAGCCG</td>
</tr>
<tr>
<td>pKP186R</td>
<td>TCGCCACCTCTGACTTGAGCC</td>
</tr>
<tr>
<td>pKP203R</td>
<td>CTAGCCTGACATCGATAAGC</td>
</tr>
<tr>
<td>7F</td>
<td>CACCACCCATATGGAACTGGCATGACAACGCACG</td>
</tr>
<tr>
<td>7R</td>
<td>GGAAAGATGCTACGGGAACTTGCTGTCCAG</td>
</tr>
<tr>
<td>8F</td>
<td>GGAATGGCGTACGTGTGGACGCCAG</td>
</tr>
<tr>
<td>8R</td>
<td>TGCATGCATCCAGGCTAGGCTAGTCC</td>
</tr>
<tr>
<td>Hel_5_FLO</td>
<td>ROX-CATGGGCACTTAGGAGATCTACGATCCCCTGGATCTCCTAGAGT</td>
</tr>
<tr>
<td>Hel_3_BHQ-2</td>
<td>TGAGATCTCTCAGTGCCATG-BHQ-2</td>
</tr>
<tr>
<td>Capture</td>
<td>CATGGGCACCTAGGAGATCTCA</td>
</tr>
<tr>
<td>5′Bio</td>
<td>(C)B(T)CATATAGCTGGACGTACATGACGATCAG</td>
</tr>
</tbody>
</table>

*In oligonucleotides used for site-directed mutagenesis, base changes relative to the wild type are highlighted in gray. Restriction sites used for cloning are underlined. t(B), biotinylated nucleotide.*
blot analysis to confirm that they resulted from a single crossover at the uvrD2 locus. Growth of a single-crossover mutant in nonselective medium should allow a second recombination between the uvrD2 flanking region carried in the chromosome and the region carried on the integrated targeting construct, pUvrD2-targ. Selection for resistance to streptomycin would select for a second recombination event and the resulting excision of the targeting plasmid carrying the psl r gene (26). Depending on where the second recombination event occurred, this would leave either a wild-type or deletion genotype. We used a PCR-based screen to differentiate between wild-type and deletion genotypes, for which we designed PCR primers that lay outside the region used for the construction of the targeting plasmid. These primers would generate a 4-kb PCR product from the wild-type gene and a 2.6-kb PCR product from a uvrD2 deletion, while a single crossover would yield a 10-kb PCR product which we would not expect to amplify under the conditions used, allowing clear assignment of genotype. After selection for the second crossover, we found that 64/64 colonies tested carried a wild-type copy of the gene. This result suggested that uvrD2 might be essential and that deletion of the gene was lethal.

To confirm this, we constructed a merodiploid strain by taking a strain carrying a single-crossover mutant of the uvrD2 targeting construct and integrating a copy of pAW24 (which carries an intact copy of uvrD2) into the L5 phage attachment site. As a control, we also integrated an empty copy of pKP186, the vector from which pAW24 was derived. Plating of exponential-phase cultures of these strains onto plates containing streptomycin selected for the second crossover events. Colonies isolated from either the merodiploid strain or the strain carrying the empty vector were then screened by PCR to check for the genotype. When pKP186 was integrated, 100% (28/28 colonies) of the colonies had a wild-type genotype, while when pAW24 was integrated, 44% (15/34 colonies) of the colonies had a uvrD2 deletion (Fig. 1). Deletion of the chromosomal copy of uvrD2 was confirmed by Southern blot analysis. These results demonstrate that uvrD2 is an essential gene in M. tuberculosis, which correlates with observations in M. smegmatis.

The tetracycline and HRDC domains of UvrD2 are dispensable. Despite our observation and the previous prediction that uvrD2 is essential in M. tuberculosis, another report described the isolation of a strain of M. tuberculosis CDC1551 carrying a transposon in uvrD2 (12). The insertion point of the transposon was identified as being 1,954 nucleotides into the gene, which lies in the region encoding the C-terminal HRDC domain of the translated protein.

We hypothesized that the conflicting results regarding the essentiality of uvrD2 might be due either to inherent strain variation between M. tuberculosis 1424 and CDC1551 or, more likely, to the fact that the C-terminal portion of UvrD2 is not required and that the N-terminal region upstream of the transposon would be expressed in the transposon mutant and be sufficient for survival. To investigate this, we decided to use complement switching (20, 32) to identify which protein domains of UvrD2 are essential. This technique exploits the dual integration/excision function of the L5 phage integrase (21, 22), whereby integrated vectors can be excised from the attachment site at a low frequency, regenerating the attB site and allowing subsequent integration of attP-containing plasmids.

To do this, we created alternate complementing constructs in a vector which also inserts into the L5 phage attachment site but which carries a gentamicin rather than a kanamycin resistance marker. By transforming the complemented uvrD2 deletion strain and selecting for Gm r Kan r colonies, it was possible to select for colonies in which the original integrating construct had been replaced by an alternate complement.

Constructs were made to contain the full-length uvrD2 gene (pAW26) or truncated versions of the gene lacking just the HRDC domain coding sequence (pAW28, containing the coding sequence for amino acids 1 to 640) or both the tetracycline and HRDC domain coding sequences (pAW27, containing the coding sequence for amino acids 1 to 600) (Fig. 2). Table 3 shows the frequencies of complement switching. Transformation of the empty vector pKP203 into the complemented uvrD2 deletion strain gave only 26 colonies with gentamicin resistance, and all of these remained kanamycin resistant, suggesting that they resulted from a random integration of the vector. Transformation with plasmids carrying the wild-type (pAW26) or truncated (pAW27 and pAW28) versions of the uvrD2 gene resulted in similar numbers of Gm r Kan r colonies, suggesting that in each case the complement had been switched. To confirm this, we designed a PCR-based screen which can distinguish between the two complements. A PCR primer designed internal to uvrD2 was used in conjunction with PCR primers designed for each vector (pKP186 or pKP203) and confirmed that we had true complement switching, with at least 12 individual colonies checked for each replacement. The replacement of the integrated uvrD2 gene with the truncated uvrD21–600 gene demonstrates that the essential functions carried out by UvrD2 can be provided by the UvrD domain.

The helicase activity of UvrD2, but not its ATPase and DNA translocase activities, is dispensable. Since the NER pathway is dispensable in mycobacteria (5, 30), the fact that UvrD2 is essential suggests that it plays a role outside NER. Because some functions of UvrD homologues do not require helicase or ATPase activity (1, 15), we investigated whether these functions are required by UvrD2.

To test this, we created mutated complementing constructs that should lack these activities by identifying important residues for each activity. The highly conserved glutamine residue in motif III of the UvrD domain is required for ATP hydrolysis in many UvrD homologues (1, 7), while a recent report has shown that changing a glutamate residue in motif V of M. smegmatis UvrD1 can abolish helicase activity while leaving ATP hydrolysis unaffected (29). Therefore, the corresponding Q266R and E508A mutations were made separately in pAW26 to give pAW29 and pAW30, respectively.

Transformation with pAW29 gave no colonies with true replacements, indicating that ATPase activity is necessary for the function of UvrD2 that renders it essential. In contrast, switched complements were obtained with pAW30, although with a lower efficiency than with the full-length or truncated constructs (Table 3). A proportion of the switched colonies were confirmed by PCR screening as described above. The replacement of the complement with the UvrD2 E508A construct shows that the helicase activity of UvrD2 is not required.

These results were confirmed by transforming the constructs bearing the point mutations, along with appropriate wild-type and vector controls, into the single-crossover mutant of the
The uvrD2 targeting construct to create merodiploid strains carrying the mutant or wild-type allele in the attB locus. Selection for double-crossover transformants resulted only in the wild-type genotype at the chromosomal locus upon complementation with UvrD2 Q266R (data not shown), like the case for the vector control, indicating that ATPase activity is required. In contrast, when strains were complemented with UvrD2 E508A, 44% of the isolated colonies were deleted for uvrD2 at its native locus, confirming that helicase activity is not necessary for the essential function of UvrD2.

To confirm that the point mutations tested affected the UvrD2 protein as we predicted, we expressed and purified wild-type UvrD2 and UvrD2 carrying the same mutations as histidine-tagged proteins in E. coli (Fig. 3). Helicase, ATPase, and DNA translocase assays were then performed using the purified proteins.

Helicase activity was measured using a fluorometric assay whereby the unwinding of the synthetic DNA substrate results in the physical separation of a fluorophore from a quencher moiety, resulting in increased fluorescence. Wild-type or mutant UvrD2 proteins were incubated at 37°C with the fluorophore/quencher-labeled DNA substrate to allow DNA binding, followed by the addition of a capture strand (corresponding in sequence to the oligonucleotide with the quencher moiety but lacking the adaptation) to prevent reannealing of separated substrate strands. Reactions were started by the addition of ATP, and DNA unwinding was measured as an increase in fluorescence.

FIG. 1. Construction of uvrD2 mutant strains. (A) Schematic showing the chromosomal locus of uvrD2 (1), the complementing copy of uvrD2 integrated into the phage attachment site (2), and the single-crossover integrated pUvrD2-targ construct (3). Checkered boxes represent the flanking regions cloned into pUvrD2-targ. A second recombination event between the flanking regions could lead to either (i) the restoration of the wild-type locus or (ii) uvrD2 deletion (4). P, PstI sites. (B) Chromosomal DNAs from the wild-type (lane 1), uvrD2 single-crossover (lane 2), and uvrD2 single-crossover merodiploid (lane 3) strains and from two ∆uvrD2 complemented strains (lanes 4 and 5) were extracted, digested with PstI, and analyzed by Southern blotting with a 32P-labeled probe corresponding to the first 300 bp of uvrD2 (shown by a black bar in panel A). (C) PCR screen showing that deletion of the chromosomal copy of uvrD2 can be achieved only when a second copy of uvrD2 is present. Upon integration of the empty vector pKP186, all double-crossover colonies isolated had a wild-type copy of uvrD2, shown by the PCR product of ~4 kb (upper panel). In the presence of a second copy of uvrD2 carried on pAW24 (lower panel), deletion of the chromosomal gene could be achieved, shown by the PCR product at ~2.6 kb (lanes 3, 4, 5, 10, 11, and 14). Lanes with no PCR product were rescreened by PCR to confirm the genotype and were found to show the wild-type PCR pattern. Lanes M, Hyperladder I (Bioline) molecular size marker.
similar extents by both ssDNA and dsDNA, and the affinity for ssDNA ($K_{DNA}^{ss}$, 0.049 ± 0.018 μM) was similar to that reported for UvrD1 ($K_{DNA}^{ss}$, 0.045 ± 0.004 μM) for oligonucleotides of equivalent length (4). We investigated different buffer conditions, DNA substrates, and metal ion specificities to see if the rate of ATP hydrolysis could be improved; however, the conditions reported appear to be optimal. It is not possible to compare these data with $M. smegmatis$ UvrD2, as only endpoint values for ATP hydrolysis were reported (31). UvrD2 E508A had ATPase activity with similar steady-state kinetics ($K_{cat}$, 66.7 μM; $V_{max}$, 0.031 s$^{-1}$) to those of the wild-type protein, with a similar DNA affinity ($K_{DNA}$, 0.045 ± 0.039 μM), while UvrD2 Q266R had no detectable ATPase activity.

The 3'-5' translocation of UvrD2 along ssDNA was investigated by its ability to displace streptavidin from a biotinylated oligonucleotide. Wild-type and mutant UvrD2 proteins were incubated with the DNA substrate in the presence of ATP, Mg$^{2+}$, and biotin to trap the displaced streptavidin. Reactions were quenched, followed by native polyacrylamide gel electrophoresis to separate free oligonucleotides from streptavidin-bound oligonucleotides. UvrD2 and UvrD2 E508A were able to displace streptavidin from the 3'-biotinylated oligonucleotide in an ATP-dependent manner, suggesting that they are able to translocate along the ssDNA substrate, while UvrD2 Q266R showed no such activity (Fig. 3D). In order to exclude the possibility that the Q266R mutation affected protein folding, therefore explaining the lack of activity we observed, we analyzed the recombinant proteins by circular dichroism. UvrD2 and UvrD2 Q266R gave absorption spectra which were indistinguishable (data not shown), showing that the effects of the Q266R mutation on protein function were not caused by protein misfolding.

Thus, the point mutations introduced affected the enzymatic activities of UvrD2 as expected, validating the conclusions reached from the genetic experiments.

**DISCUSSION**

In this study, we demonstrated conclusively that $uvrD2$ is essential in $M. tuberculosis$ and determined which of its properties are required for its essential function.

**TABLE 3. Vector switching frequencies of $uvrD2$ alleles**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>No. of Gm$\text{'}$ transformants $^a$</th>
<th>No. of Kmr$\text{'}$ colonies/no. of transformants $^a$</th>
</tr>
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<tbody>
<tr>
<td>pAW26</td>
<td>$uvrD2$</td>
<td>144 ± 43</td>
<td>58/64</td>
</tr>
<tr>
<td>pAW27</td>
<td>$uvrD2_{1-600}$</td>
<td>152 ± 10</td>
<td>51/64</td>
</tr>
<tr>
<td>pAW28</td>
<td>$uvrD2_{1-600}$</td>
<td>163 ± 50</td>
<td>55/64</td>
</tr>
<tr>
<td>pAW29</td>
<td>$uvrD2E508A$</td>
<td>8 ± 3</td>
<td>0/25</td>
</tr>
<tr>
<td>pAW30</td>
<td>$uvrD2E508A$</td>
<td>90 ± 33</td>
<td>47/64</td>
</tr>
<tr>
<td>pKP203</td>
<td>$uvrD2E508A$</td>
<td>9 ± 3</td>
<td>0/28</td>
</tr>
</tbody>
</table>

$^a$ The complemented $\Delta uvrD2$ strain was transformed with 500 ng of the indicated plasmid and with 300 ng PBS-Int. Colonies were selected for gentamicin resistance and then tested for kanamycin sensitivity.

$^b$ Data shown are means ± standard deviations for gentamicin-resistant transformants obtained from three independent transformations from two separate experiments.

$^c$ The number of kanamycin-sensitive colonies, indicating true complement replacement, is reported. Twelve kanamycin-sensitive colonies from each successful switch were verified by PCR and sequencing, and all showed that they were true complement replacements.
Neither the HRDC domain nor the tetracysteine module was needed, providing an explanation for the reported isolation of a transposon mutant of *uvrD2*, as the transposon is located in the region encoding the HRDC domain (12). Biochemical studies of *M. smegmatis* UvrD2 (31) have indicated that deletion of the HRDC domain alone does not significantly affect ATPase or helicase activity, whereas the additional removal of the tetracysteine domain eliminates helicase but not ATPase activity. Taking this into account, our observations might suggest that helicase activity is not required for the essential role of UvrD2. However, Sinha et al. (31) found that the addition of Ku, a component of the nonhomologous end-joining DNA repair system, restored helicase function to the truncated UvrD2 protein, and Ku would be present in our genetic experiments.

By introducing point mutations into conserved motifs, we were able to separate these two biochemical properties of UvrD2. One mutation (Q266R) eliminated all detectable activity, while the other (E508A) abolished helicase activity without eliminating the ability of UvrD2 to hydrolyze ATP in a DNA-dependent manner or to translocate along DNA. It remains possible that Ku could enable helicase activity of UvrD2 E508A, but this is unlikely; the ability of Ku to stimulate DNA unwinding by the truncated *M. smegmatis* UvrD2 protein was investigated on the basis that Ku could stimulate helicase activity in *M. smegmatis* UvrD1 (30), yet Ku could not stimulate helicase activity in *M. smegmatis* UvrD1 carrying an E609A mutation, the mutation corresponding to that in *M. tuberculosis* UvrD2 E508A. The use of these point mutants in complementation experiments clearly demonstrated that helicase activity was not required. In contrast, the ATPase activity of UvrD2 was necessary for the bacterium to survive. The nonessentiality of helicase activity is useful information for directing screening approaches should UvrD2 be pursued as a potential new drug target for *M. tuberculosis*.

The requirement for ATP hydrolysis appears surprising given the slow kinetics observed for UvrD2. The affinity of UvrD2 for ATP (K$_{m}$, 68 μM) is similar to those seen for UvrD1 (K$_{m}$, 60.2 μM) and *E. coli* UvrD (K$_{m}$, 53 μM), while the catalytic constants differ greatly (k$_{cat}$, 0.035 s$^{-1}$ compared to 43 s$^{-1}$ and 95 s$^{-1}$, respectively). The rate of DNA unwinding shown by UvrD2 is comparable to that seen with UvrD1, suggesting that the purified recombinant protein is fully active. The lack of translocation by UvrD2 Q266R suggests that UvrD2 uses the energy from ATP hydrolysis to drive DNA translocation and that the observed slow ATP hydrolysis by
UvrD2 is sufficient to allow DNA translocation. Alternatively, it is possible that the conditions tested did not allow optimal ATP hydrolysis, although the same buffer conditions and substrates allowed DNA unwinding by UvrD2 to occur at rates comparable to those seen with other DNA helicases, and extensive attempts to identify a buffer composition which would allow an increase in the rate of observed ATP hydrolysis were unsuccessful, suggesting that these conditions allow optimal protein activity and that the observed steady-state kinetics are the true values.

The ability of UvrD2 to translocate along ssDNA and displace streptavidin suggests that UvrD2 is capable of translocating along DNA and imparting a force on a molecule blocking its path. The apparent requirement for ATP hydrolysis and/or DNA translocation, but not helicase activity, suggests that the essential function of UvrD2 is most likely to involve some such DNA translocation and, potentially, protein displacement. Mycobacterial UvrD1 can function to inhibit RecA-mediated strand exchange (28); whether UvrD2 also plays a role in regulating recombination remains to be seen.

The identification of the crucial role of UvrD2 in M. tuberculosis growth and survival will require additional studies.

ACKNOWLEDGMENTS

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