



# Emergence of *mmpT5* Variants during Bedaquiline Treatment of *Mycobacterium intracellulare* Lung Disease

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**ABSTRACT** Bedaquiline (BDQ), a diarylquinoline antibiotic that targets ATP synthase, is effective for the treatment of *Mycobacterium tuberculosis* infections that no longer respond to conventional drugs. While investigating the off-label use of BDQ as salvage therapy, seven of 13 patients with *Mycobacterium intracellulare* lung disease had an initial microbiological response and then relapsed. Whole-genome comparison of pretreatment and relapse isolates of *M. intracellulare* uncovered mutations in a previously uncharacterized locus, *mmpT5*. Preliminary analysis suggested similarities between *mmpT5* and the *mmpR5* locus, which is associated with low-level BDQ resistance in *M. tuberculosis*. Both genes encode transcriptional regulators and are adjacent to orthologs of the *mmpS5-mmpL5* drug efflux operon. However, MmpT5 belongs to the TetR superfamily, whereas MmpR5 is a MarR family protein. Targeted sequencing uncovered nonsynonymous *mmpT5* mutations in isolates from all seven relapse cases, including two pretreatment isolates. In contrast, only two relapse patient isolates had nonsynonymous changes in ATP synthase subunit *c* (*atpE*), the primary target of BDQ. Susceptibility testing indicated that *mmpT5* mutations are associated with modest 2- to 8-fold increases in MICs for BDQ and clofazimine, whereas one *atpE* mutant exhibited a 50-fold increase in MIC for BDQ. Bedaquiline shows potential for the treatment of *M. intracellulare* lung disease, but optimization of treatment regimens is required to prevent the emergence of *mmpT5* variants and microbiological relapse.

**KEYWORDS** bedaquiline, *Mycobacterium avium*, *Mycobacterium intracellulare*, antibiotic resistance

The diarylquinoline bedaquiline (BDQ; Sirturo) represents a new class of antimycobacterial agents. Primarily developed for the treatment of *Mycobacterium tuberculosis* infections, BDQ has been successfully used to treat multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (1–4) and is approved by the U.S. Food and Drug Administration (FDA) for MDR tuberculosis (TB) treatment in combination with other active tuberculosis drugs (5). BDQ is an ATP synthase inhibitor that is bactericidal for *M. tuberculosis* (6). Functional and structural studies have confirmed that diarylquinolines abolish ATP synthesis by binding to the subunit *c* rotor ring complex, encoded by *atpE* (7). Consistent with this, BDQ resistance was first associated with mutations in the *atpE* gene (8, 9). However, *atpE* mutations are rare *in vivo*, and when strains with elevated MICs to BDQ were selected *in vitro*, only 28% (15/53 strains) exhibited changes in *atpE* (10). Subsequent studies demonstrated that modest ( $\leq 8$ -fold) increases in MIC to BDQ are associated with mutations in two nontarget loci, Rv0678 (*mmpR5*) (11, 12) and Rv2535c (*pepQ*) (13). MmpR5 is a transcriptional regulator

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that controls the expression of genes encoding the MmpS5-MmpL5 efflux pump. Upregulation of MmpS5-MmpL5 (e.g., due to inactivation of MmpR5) is associated with low-level resistance to various drugs, including the antimycobacterial agent clofazimine (CLF). PepQ, a putative Xaa-Pro aminopeptidase, also confers low-level resistance to BDQ, but the underlying mechanism has not been elucidated.

Although it is FDA approved only for the treatment of MDR-TB, BDQ shows activity against many nontuberculous mycobacteria (NTM), including species of the *Mycobacterium avium* complex (MAC) (8, 9). When used alone, the drug is not bactericidal against MAC *in vitro* or *in vivo* (minimum bactericidal concentration [MBC], >128  $\mu\text{g/ml}$ ). However, when BDQ is given in combination with amikacin or amikacin plus clarithromycin, bactericidal activity is observed after 3 to 4 months of treatment (14). In 2015, the first study describing the off-label use of BDQ for the treatment of patients with refractory MDR NTM was reported by Philley et al. (15). It included six patients with MAC lung disease. All had a measurable response to therapy, and two-thirds developed one or more negative sputum culture. Unfortunately, several of the patients who initially responded to BDQ experienced microbiological relapse while on therapy. Reduced susceptibility to BDQ was suspected, but the phenotypic and genotypic characteristics of the pretreatment and relapse isolates were not assessed.

Phenotypic susceptibility testing remains the gold standard for assessing drug resistance among NTM. However, *in vitro* results do not always correlate with *in vivo* performance, and breakpoints for many drugs, including BDQ, have not yet been established (16). Nonetheless, we have recently implemented a BDQ susceptibility testing method for MAC that can identify isolates with elevated MICs (defined as MICs higher than those of untreated or wild strains) (17). Here, we examine MAC isolates recovered before, during, and after BDQ therapy and describe a novel genetic locus associated with microbiological relapse.

## RESULTS

**Patient population.** A total of 16 BDQ-treated MAC patients from University of Texas Health Science Center at Tyler (UTHSCT) were identified, including six patients previously described in the study by Philley et al. (15). Species-level identification of MAC isolates by partial 16S rRNA gene sequencing revealed that 13 of 16 cases involved *M. intracellulare*. Of these, eight patients experienced microbiological relapse, despite an initial positive response to BDQ treatment. In five cases, the acid-fast bacilli (AFB) smear and culture results became negative before relapsing to 3+/4+ levels. Patient 6 became smear negative and culture status improved (i.e., counts as low as 8 CFU on Middlebrook 7H11 agar) but remained positive. Patient 5 exhibited only minor declines in smear and culture counts, but these were considered significant, since prior to BDQ treatment, the patient had produced 20 consecutive 4+ cultures. Closer examination of the eighth case, including variable-number tandem-repeat (VNTR) analysis (18), revealed that relapse was due to a new infection with a genotypically distinct strain of *M. intracellulare* and not a mutation of the pretreatment isolate. The clinical characteristics of the seven patients who experienced true relapses are summarized in Table 1. Four patients had nodular bronchiectasis disease, and three patients had fibronodular upper-lobe disease. Five patients also had features of cavitary disease. Prior to the addition of BDQ, the patients had been on MAC treatment for 20 to 96 months. All seven patients were heavily AFB smear and culture positive, and four patients exhibited macrolide resistance. Microbiological relapse occurred after the patients had been on BDQ treatment for  $\geq 3$  months.

**Susceptibility testing.** The antibiotic susceptibility testing results for BDQ, CLF, amikacin (AMK), and clarithromycin (CLR) are summarized in Table 2. In most cases, the MICs for pretreatment strains were 0.004  $\mu\text{g/ml}$  for BDQ and  $\leq 0.06$   $\mu\text{g/ml}$  for CLF, whereas the MICs for relapse isolates were typically  $\geq 0.008$   $\mu\text{g/ml}$  for BDQ and  $\geq 0.12$   $\mu\text{g/ml}$  for CLF. The BDQ MICs of pretreatment isolates from patients 3 and 6 were 0.008  $\mu\text{g/ml}$ .

**TABLE 1** Clinical characteristics of seven *M. intracellulare* patients who responded to BDQ and then relapsed

Patient	Age (yr)	Disease type <sup>a</sup>	Duration of prior treatment (mo)	Companion drugs while on BDQ <sup>b</sup>	Resistance		AFB smear status			7H11 agar culture status		
					Macrolide	Amikacin	Pretreatment	Lowest on BDQ	Relapse	Pretreatment	Lowest on BDQ	Relapse
1 <sup>c</sup>	54	FN + C	96	EMB, RFB, STR	Yes	No	4+	Negative	3+	4+	Negative	4+
2 <sup>c</sup>	60	NB	25	AZM, EMB, RFB, STR	No	No	4+	Negative	3+	4+	Negative	4+
3	68	FN + C	48	AMK, EMB, RFB	Yes	No	3+	Negative	2+	4+	Negative	2+
4	71	NB + C		AMK, AZM, EMB, STR	No	No	3+	Negative	2+	4+	Negative	3+
5 <sup>c</sup>	58	NB	78	EMB, RFB, STR	Yes	Yes	4+	2+	4+	4+	3+	4+
6	66	NB + C	44	AMK, EMB, RFB	Yes	Yes	3+	Negative	2+	3+	+ <sup>d</sup>	3+
7	64	FN + C	20	CLR, EMB, RFB, STR	No	No	3+	Negative	3+	4+	Negative	3+

<sup>a</sup>FN, fibronodular; NB, nodular bronchiectasis; C, cavitory.

<sup>b</sup>AMK, amikacin; AZM, azithromycin; CLR, clarithromycin; EMB, ethambutol; RFB, rifabutin; STR, streptomycin.

<sup>c</sup>Previously described by Philley et al. (15).

<sup>d</sup>Only 8 CFU were observed.

**TABLE 2** Antibiotic susceptibility profiles and genotypic characteristics of pretreatment and relapse isolates of *M. intracellulare*<sup>a</sup>

Isolate by patient	Treatment status (mo)	MIC ( $\mu\text{g/ml}$ ) <sup>b</sup>		Susceptibility <sup>c</sup>			MmpT5 sequence <sup>d</sup>	AtpE sequence <sup>d</sup>
		BDQ	CLF	AMK	CLR	<i>mmpT5</i> allele		
<b>1</b>								
1A	Pretreatment	0.004	$\leq 0.06$	R	R	3.1	WT	WT
1B	On BDQ (5)	0.008	0.12	R	R	3.2	Gly <sup>66</sup> →fs	WT
1F	Post-BDQ (3)	0.004	$\leq 0.06$	R	R	3.3	Arg <sup>25</sup> →Pro	Ala <sup>65</sup> →Pro
<b>2</b>								
2A	Pretreatment	0.004	$\leq 0.06$	R	S	7.1	WT	WT
2C	On BDQ (9)	0.03	$\leq 0.06$	R	S	7.2	Val <sup>46</sup> →Gly	WT
2D	Post-BDQ (6)	0.03	0.12	R	S	7.2	Val <sup>46</sup> →Gly	WT
<b>3</b>								
3B	Pretreatment	0.004	0.12	S	S	5.1	Glu <sup>177</sup> →Lys	WT
3F	On BDQ (10)	0.008	0.25	R	S	5.2	Ala <sup>23</sup> →Pro + Glu <sup>177</sup> →Lys	WT
3G	On BDQ (11)	0.008	0.5	R	S	5.2	Ala <sup>23</sup> →Pro + Glu <sup>177</sup> →Lys	WT
3H	On BDQ (15)	0.008	$\leq 0.06$	R	S	5.1	Glu <sup>177</sup> →Lys	WT
<b>4</b>								
4A	Pretreatment	0.004	$\leq 0.06$	S	S	4.1	WT	WT
4C	On BDQ (4.5)	0.015	0.25	S	S	4.2	Pro <sup>104</sup> →fs	WT
<b>5</b>								
5A	Pretreatment	0.004	$\leq 0.06$	R	R	1.1	WT	WT
5C	On BDQ (8)	0.004	$\leq 0.06$	R	R	1.2	Ala <sup>162</sup> →Pro	WT
5D	On BDQ (15)	0.008	$\leq 0.06$	R	R	1.2	Ala <sup>162</sup> →Pro	WT
5E	On BDQ (23)	2	ND	R	R	1.1	WT	Ala <sup>65</sup> →Pro
5F	On BDQ (28)	2	$\leq 0.06$	R	R	1.1	WT	Ala <sup>65</sup> →Pro
<b>6</b>								
6A	Pretreatment	0.008	0.12	R	R	5.1	Glu <sup>177</sup> →Lys	WT
6B	On BDQ (7)	0.008	0.25	R	R	5.3	Ile <sup>19</sup> →Ser + Glu <sup>177</sup> →Lys	WT
6C	Post-BDQ (4.5)	0.008	0.25	R	R	5.4	Val <sup>35</sup> →Gly + Glu <sup>177</sup> →Lys	WT
6E	Post-BDQ (8.5)	0.03	0.5	R	R	5.1	Glu <sup>177</sup> →Lys	WT
<b>7</b>								
7A	Pretreatment	0.004	$\leq 0.06$	S	S	7.1	WT	WT
7C	Post-BDQ (5)	0.015	0.5	S	S	7.3	Pro <sup>104</sup> →fs	WT
7D	Post-BDQ (5)	0.03	0.5	S	S	7.3	Pro <sup>104</sup> →fs	WT
7E	Post-BDQ (10)	0.03	0.5	S	S	7.3	Pro <sup>104</sup> →fs	WT

<sup>a</sup>For the complete list of pretreatment and relapse isolates, see Table S1.<sup>b</sup>CLF, clofazimine; ND, not determined.<sup>c</sup>AMK, amikacin; CLR, clarithromycin; R, resistant; S, susceptible.<sup>d</sup>WT, wild-type sequence; fs, frameshift mutation.

**Whole-genome sequencing.** Whole-genome sequencing of five *M. intracellulare* isolates (1A and 1B from patient 1, and isolates 2A, 2B, and 2C from patient 2) generated a median number of 2.25 million reads per isolate, at a median depth of 120-fold coverage. Reference-based mapping of sequencing data to available MAC genomes confirmed that the strains were *M. intracellulare*. However, the comparison also revealed extensive genetic diversity (e.g., insertions, deletions, and single nucleotide polymorphisms) among the sequenced and reference genomes, which precluded the use of a reference mapping approach for the identification of candidate BDQ resistance loci. Instead, *de novo* contig assemblies were generated for all 5 strains. Sequencing reads were mapped to these *de novo* draft genomes, and high-quality single nucleotide variants (SNVs) were identified. The pretreatment and relapse isolates from patient 1 differed by only five high-quality SNVs (variant frequency, >95%; quality = 35). A comparison of strains from patient 2 revealed six SNVs, with five SNVs shared by both postrelapse isolates (i.e., 2B and 2C) and one SNV unique to isolate 2C. No *atpE* mutations were found, and no SNVs were shared by all five isolates. However, all three relapse isolates (i.e., 1B, 2B, and 2C) had nonsynonymous mutations in a previously uncharacterized open reading frame adjacent to the *mmpS5-mmpL5* operon. This locus

**TABLE 3** Allelic diversity of *mmpT5* among clinical isolates of *M. intracellulare*

<i>mmpT5</i> position	<i>mmpT5</i> allele <sup>a</sup>																	Nucleotide change	Status <sup>b</sup>	Amino acid change
	1.1	1.2	2.1	3.1	3.2	3.3	4.1	4.2	5.1	5.2	5.3	5.4	6.1	7.1	7.2	7.3	8.1			
56	T	●	●	●	●	●	●	●	●	●	G	●	●	●	●	●	●	T <sub>56</sub> →G	NS	Ile <sup>19</sup> →Ser
67	G	●	●	●	●	●	●	●	●	●	C	●	●	●	●	●	●	G <sub>67</sub> →C	NS	Ala <sup>23</sup> →Pro
72	A	●	●	●	●	●	●	●	●	●	●	●	G	G	G	G	G	A <sub>72</sub> →G	Syn	Val <sup>24</sup> →Val
74	G	●	●	●	●	C	●	●	●	●	●	●	●	●	●	●	●	G <sub>74</sub> →C	NS	Arg <sup>25</sup> →Pro
104	T	●	●	●	●	●	●	●	●	●	●	●	G	●	●	●	●	T <sub>104</sub> →G	NS	Val <sup>35</sup> →Gly
129	A	●	●	●	●	●	G	G	●	●	●	●	●	●	●	●	●	A <sub>129</sub> →G	Syn	Glu <sup>43</sup> →Glu
137	T	●	●	●	●	●	●	●	●	●	●	●	●	●	G	●	●	T <sub>137</sub> →G	NS	Val <sup>46</sup> →Gly
196/197		●	●	●	C	●	●	●	●	●	●	●	●	●	●	●	●	C <sub>196_197</sub> →insC <sup>c</sup>	FS	Gly <sup>66</sup> →fs
311/312		●	●	●	●	●	●	C	●	●	●	●	●	●	●	●	●	C <sub>311_312</sub> →insC	FS	Pro <sup>104</sup> →fs
378	G	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	A	G <sub>378</sub> →A	Syn	Ala <sup>126</sup> →Ala
429	C	●	●	●	●	●	●	●	●	●	●	●	●	T	T	T	T	C <sub>429</sub> →T	Syn	Val <sup>143</sup> →Val
450	C	●	●	●	●	●	●	●	●	●	●	●	●	T	●	●	●	C <sub>450</sub> →T	Syn	Pro <sup>150</sup> →Pro
471	G	●	●	A	A	A	A	A	A	A	A	●	●	●	●	●	●	G <sub>471</sub> →A	Syn	Leu <sup>157</sup> →Leu
484	G	C	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	G <sub>484</sub> →C	NS	Ala <sup>162</sup> →Pro
504	G	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	G <sub>504</sub> →Z	Syn	Ser <sup>168</sup> →Ser
522	G	●	●	●	●	●	●	●	●	●	●	●	●	A	A	A	●	G <sub>522</sub> →A	Syn	Ala <sup>174</sup> →Ala
528	C	●	T	T	T	T	T	T	T	T	T	T	T	T	T	T	●	C <sub>528</sub> →T	Syn	Gly <sup>176</sup> →Gly
529	G	●	●	●	●	●	●	●	A	A	A	A	●	●	●	●	●	G <sub>529</sub> →A	NS	Glu <sup>177</sup> →Lys
582	G	●	●	●	●	●	●	●	●	●	●	●	●	A	A	A	●	G <sub>582</sub> →A	Syn	Ala <sup>194</sup> →Ala

<sup>a</sup>● identical to allele 1.1 (*mmpT5* sequence from *M. intracellulare* ATCC 13950<sup>T</sup>).

<sup>b</sup>Syn, synonymous change; NS, nonsynonymous change; FS, frameshift mutation.

<sup>c</sup>insC, insertion of C.

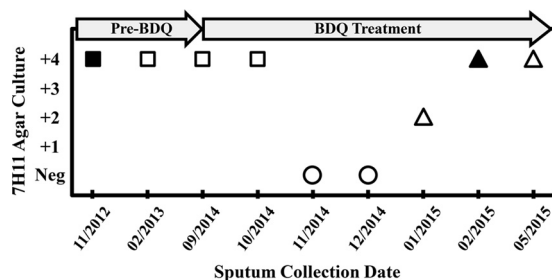
is predicted to encode a transcriptional regulator and control expression of the *mmpS5-mmpL5* operon. Notably, this regulator is not a homolog of *MmpR5*, which occupies the equivalent position in the *M. tuberculosis* genome. To emphasize this distinction, we have named the MAC locus *mmpT5*.

**Targeted gene sequencing. (i) *mmpT5*.** A phylogenetic comparison of *mmpT5* gene sequences from MAC type and reference strains indicates that *M. intracellulare* sequences are most similar to those from *Mycobacterium chimaera*, *Mycobacterium yongonense*, and the recently validated MAC species *M. paraintracellulare* (19), whereas sequences from *M. avium* and *Mycobacterium colombiense* are more distant (Fig. S1A). To assess the variability of *mmpT5* among clinical isolates, targeted gene sequencing was performed on 50 *M. intracellulare* isolates from patients in our study group (Table S1). A comparison with *M. intracellulare* ATCC 13950<sup>T</sup> revealed 19 variable sites, including 10 sites associated with synonymous substitutions and 9 sites with nonsynonymous mutations (Table 3). Among pretreatment isolates, 8 different *mmpT5* alleles were identified. These differed by 0 to 5 single nucleotide variants (SNVs) from the ATCC 13950<sup>T</sup> *mmpT5* gene (Tables 3 and S1, and Fig. S1B). With the exception of G<sub>529</sub>→A (Glu<sup>177</sup>→Lys), which was present in all isolates from patients 3 and 6, the pretreatment alleles only contained synonymous substitutions relative to the ATCC 13950<sup>T</sup> *mmpT5* sequence. In contrast, isolates associated with microbiological relapse contained nonsynonymous mutations. Most SNVs were restricted to individual patients, although the same frameshift (fs) mutation (G<sub>311\_312</sub>→insC; Pro<sup>104</sup>→fs) occurred independently in patients 4 and 7 (Tables 2, 3, and S1).

**(ii) *atpE*.** Pretreatment isolates from all 13 patients with *M. intracellulare* infections had wild-type *atpE* sequences. Among relapse cases, *atpE* variants were observed only twice. Three months after the end of BDQ treatment, isolates containing mutations in both *mmpT5* (G<sub>74</sub>→C and Arg<sup>25</sup>→Pro) and *atpE* (G<sub>221</sub>→C and Ala<sup>65</sup>→Pro) were recovered from patient 1. After 2 years of BDQ treatment, multiple isolates with the same *atpE* mutation (*atpE* G<sub>221</sub>→C and Ala<sup>65</sup>→Pro) were recovered from patient 5 (Tables 2 and S1).

## DISCUSSION

Bedaquiline (BDQ) is a welcome addition to the antimycobacterial arsenal. Despite initial concerns about side effects (especially cardiac toxicity), BDQ-containing regimens



**FIG 1** Representative microbiological response of a patient to BDQ treatment. Sputum samples for *M. intracellulare* culture were collected on the dates indicated (month/year). Targeted sequencing of *mmpT5* was performed on positive cultures. Circles, negative, no AFB recovered from agar culture; squares, wild-type *mmpT5* allele; triangles, mutant *mmpT5* allele. MICs (in micrograms per milliliter) were determined for ■ (BDQ, 0.004; CLF,  $\leq 0.06$ ) and ▲ (BDQ, 0.008; CLF, 0.12).

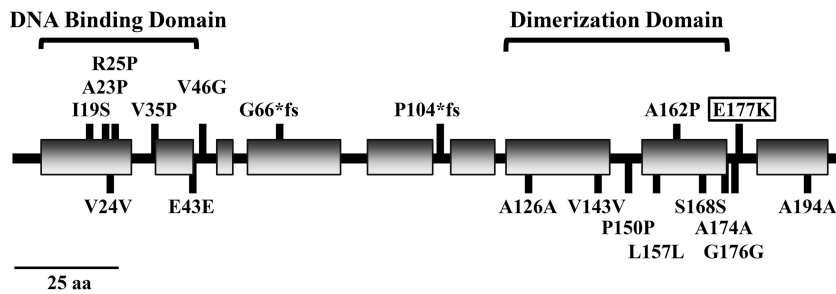
have proven to be well tolerated and efficacious for the treatment of drug-resistant *M. tuberculosis* infections (4). For patients with NTM infections, preliminary findings are promising, but more studies are required. In a recent uncontrolled study of patients with MAC or *Mycobacterium abscessus* lung disease refractory to prior drug therapy, the off-label use of BDQ was well tolerated. No serious adverse effects or abnormal electrocardiogram (ECG) changes occurred (15). Five of the six MAC patients showed symptomatic improvement (e.g., less cough, less sputum production, and/or improved energy level). None achieved sputum conversion (i.e., three consecutive AFB-negative cultures) during the 6-month study period, but four patients developed one or more negative cultures (i.e., no growth on Middlebrook 7H11 agar) despite having heavily positive (4+) pretreatment cultures. Four of the MAC patients eventually experienced microbiological relapse. Unfortunately, Phillely et al. (15) were unable to perform therapeutic drug monitoring or antibiotic susceptibility testing or evaluate the possibility of emerging BDQ resistance among relapse isolates. In the current study, we revisited the issue of microbiological relapse. *M. intracellulare* isolates associated with microbiological relapse after  $\geq 3$  months of therapy exhibited elevated MICs for BDQ. Modest (i.e., 2- to 8-fold) elevation of MICs to BDQ and CLF is associated with the acquisition of nonsynonymous mutations in the previously uncharacterized gene *mmpT5*.

Of the 16 patients initially enrolled in this study, 13 patients had lung disease due to *M. intracellulare*, including seven who experienced true microbiological relapse. The experience of a typical patient is shown in Fig. 1. Despite combination therapy with multiple drugs, sputum cultures were heavily positive for *M. intracellulare* throughout the 20 months prior to BDQ initiation. Following the addition of BDQ, two consecutive specimens were culture negative on Middlebrook 7H11 agar, but this response was short-lived. Microbiological relapse was associated with a  $\geq 2$ -fold increase in MICs for BDQ and CLF. Analysis of the relapse strain revealed a mutation in the *mmpT5* gene.

Pretreatment MICs of *M. intracellulare* strains for BDQ were 0.004  $\mu\text{g/ml}$  (six patients) to 0.008  $\mu\text{g/ml}$  (one patient; see Table 2). This range is consistent with the results of a recent study conducted by members of our group (17). Briefly, of 103 MAC isolates tested by broth microdilution, 90 (87%) isolates showed BDQ MICs of  $\leq 0.008 \mu\text{g/ml}$ , and only one isolate had an MIC of  $\geq 0.015$ . These values are also similar to the 0.007 to 0.010  $\mu\text{g/ml}$  range reported for 7 MAC strains using a Bactec system but lower than the  $\geq 0.03 \mu\text{g/ml}$  reported for BDQ-susceptible *M. tuberculosis* strains (8, 20). After treatment, *M. intracellulare* isolates with elevated MICs were obtained from all relapse patients. We recognize that there is some variability associated with *in vitro* susceptibility testing and that 2-fold changes in MIC must be interpreted with caution. However, when assessing serial isolates from individuals on BDQ treatment, even small MIC differences may be relevant.

Loci associated with BDQ resistance have been reported for *M. tuberculosis* but not previously described for the MAC. The  $F_0F_1$  ATP synthase subunit *c* targeted by BDQ is conserved in the MAC, but only a subset of BDQ-resistant *M. tuberculosis* strains contain





**FIG 2** Features of MmpT5. Protein modeling predicts that MmpT5 belongs to the TetR superfamily of transcriptional regulators. It features an N-terminal DNA-binding domain and a C-terminal dimerization domain. Shaded rectangles represent predicted  $\alpha$ -helical regions. Mutations associated with *M. intracellulare* pretreatment, and relapse isolates are shown. Mutations were found throughout MmpT5, but the majority of nonsynonymous substitutions occur in the DNA-binding domain. The E177K substitution (boxed) at the edge of the predicted dimerization domain was present in pretreatment isolates from two patients.

*atpE* mutations (10). Comparative genomics indicate that the Rv0678 (*mmpR5*) locus, which is associated with low-level BDQ resistance in *M. tuberculosis* (11, 12), has no ortholog in the MAC. In order to identify *M. intracellulare* loci associated with elevated BDQ MICs, pretreatment and relapse isolates from two patients were subjected to whole-genome sequencing. Due to the extensive variability that is characteristic of MAC strains (21, 22), it was not feasible to identify candidate loci by comparing these sequences with publicly available *M. intracellulare* genomes. Instead, a *de novo* genome assembly approach was used. Sequences from isolates 1A and 1B (i.e., strain BDQ1) are most similar to the genome of *Mycobacterium* sp. strain MOTT36Y (NC\_017904), whereas the 2A, 2B, and 2C isolates (i.e., strain BDQ2) appear to represent a different lineage of *M. intracellulare*. The BDQ1 strain is predicted to contain a large (~100-kb) plasmid that is similar to a conjugative plasmid that is widely distributed among other NTM species, including the MAC (23). A comparison of the BDQ1 pretreatment and relapse isolates revealed five SNVs. Among the BDQ2 isolates, five SNVs were shared by both relapse isolates, and a sixth SNV was only found in the 2C genome. Although no SNVs were shared by all three relapse isolates, all had mutations in an uncharacterized locus upstream of the *mmpS5-mmpL5* operon. Expression of the MmpS5-MmpL5 efflux system is associated with low-level resistance to antibiotics, including BDQ, CLF, and azole compounds (24). In *M. tuberculosis*, overexpression of MmpS5-MmpL5 can be caused by mutations in MmpR5, a MarR family transcriptional regulator that is encoded by the Rv0678 locus, which is adjacent to *mmpS5-mmpL5*. As previously indicated, *M. intracellulare* does not possess an ortholog of MmpR5. Instead, sequence analysis indicates that the uncharacterized *M. intracellulare* locus encodes a transcriptional regulator of the TetR family. The position of this gene and its association with BDQ resistance suggests that it controls the MAC *mmpS5-mmpL5* operon. Because of its predicted role in transcription of the *mmp5* operon, we have named this previously uncharacterized gene *mmpT5*. Experiments to formally assess the role of this regulator on *mmpS5-mmpL5* transcription are under way.

Targeted sequencing of clinical *M. intracellulare* strains revealed the *mmpT5* gene to be polymorphic (Tables 3 and S1). Analysis of pretreatment isolates uncovered eight BDQ alleles composed of 10 synonymous and 1 nonsynonymous SNV. Eight additional polymorphisms, including six nonsynonymous SNVs and two frameshift mutations, were identified through sequencing of relapse isolates. TetR family regulators are homodimers. Each  $\alpha$ -helical monomer features an N-terminal DNA-binding domain and a C-terminal dimerization/regulatory region (25). Protein modeling with Phyre2 (26) and JPred4 (27) predicts that five of the nonsynonymous SNVs are located in the DNA-binding domain of MmpT5, and two SNVs are located in the dimerization domain (Fig. 2). Mutations in these domains may impair MmpT5 homodimer formation and repression of (i.e., binding to) the *mmpS5-mmpL5* operon, but functional studies are required

to define roles for the individual substitutions. The frameshift mutations, which are predicted to abrogate MmpT5 activity, are consistently associated with increased MICs for both BDQ and CLF.

Although *mmpT5* mutations were common among relapse isolates, *atpE* mutations were only observed in two cases. Isolates containing mutations in both *mmpT5* (G<sub>74</sub>→C; Arg<sup>25</sup>→Pro) and *atpE* (G<sub>221</sub>→C; Ala<sup>65</sup>→Pro) were obtained from patient 1, and isolates that were wild type for *mmpT5* but exhibited the *atpE* G<sub>221</sub>→C (Ala<sup>65</sup>→Pro) mutation were recovered from patient 5 (Tables 2 and S1). The *M. intracellulare* mutation is equivalent to the Ala<sup>63</sup>→Pro change, which has been observed in BDQ-resistant *M. tuberculosis* strains (28). In contrast to *mmpT5* mutations, which appeared in these patients within five (patient 1) to 8 months (patient 5) of BDQ initiation, the *atpE* mutations emerged much later, i.e., after patient 1 stopped therapy, and almost 2 years into patient 5's BDQ treatment.

BDQ is a promising agent for the treatment of MAC lung disease. In this study, even patients who eventually experienced microbiological relapse initially showed clinical improvement and reduced AFB burdens. Of concern, *M. intracellulare* isolates with new *mmpT5* mutations and elevated MICs emerged within 3 to 8 months, much more rapidly than has been observed in MDR-TB trials. The relapse isolates showed only modest increases (i.e., ≤8-fold) in BDQ MICs, and *in vitro* values were ≤0.03 μg/ml, much lower than the BDQ concentrations expected to be maintained during therapy. For example, Diacon et al. (29) treated MDR-TB patients with a regimen that included thrice-weekly doses of 200 mg of BDQ. Throughout the dosing period, serum levels of BDQ remained above 0.6 μg/ml. Therapeutic drug monitoring was not available for our patients, but an equivalent dosing strategy was used. Additional studies are required to determine if this phenomenon of microbiological relapse is widespread during BDQ treatment of MAC disease, more common with *M. intracellulare* infections, or restricted to the challenging MDR strains found in our patient population.

A confounding factor may be the use of rifabutin (RFB) as a companion drug. Rifamycins induce the CYP450 system, which is the pathway for metabolism of BDQ. Thus, RFB cotreatment may compromise therapy by accelerating drug clearance and lowering BDQ exposure (30). Notably, of the seven cases with microbiological relapse, six were cotreated with RFB. Relapse isolates from those patients had elevated BDQ MICs and *mmpT5* mutations, but the highest value (MIC, 2 μg/ml) was associated with an *atpE* mutant obtained from patient 5, who did not receive RFB. The impact of companion drugs and dosing schedules on the clinical efficacy of BDQ deserves further investigation, as the optimization of BDQ-containing regimens may slow or prevent the emergence of *mmpT5* variants and improve treatment outcomes for all patients with MAC lung disease.

## MATERIALS AND METHODS

**Patients.** Patients for this study were identified from the NTM/Bronchiectasis Clinic of the University of Texas Health Science Center at Tyler (UTHSCT). All had treatment-refractory MDR-MAC lung disease and were approved for off-label therapy with BDQ. The treatment details for some patients have been presented previously (15). For the first 2 weeks of treatment, BDQ was dosed at 400 mg per day. The dose was then decreased to 200 mg three times per week. The BDQ treatment period and routine acid-fast bacilli (AFB) sputum smear and culture results were collected from chart reviews (Table 1). A decline in AFB smear and culture statuses was interpreted as a microbiological response to BDQ treatment. A patient was considered to have experienced a microbiological relapse if, after an initial response to treatment, their AFB smear and culture (i.e., CFU counts on Middlebrook 7H11 agar) statuses increased.

**Informed consent.** Retrospective chart reviews and molecular analysis (i.e., DNA sequencing) of patient isolates were approved by the institutional review board at UTHSCT.

**Bacterial strains.** Patients undergoing treatment for MAC infection were asked to submit sputum samples once per month. Samples were processed and cultured on liquid and solid media using standard methods (31) and then stored at -70°C or maintained at room temperature (20 to 22°C) in their original broth culture bottles. Cultures were identified as MAC using AccuProbe tests (Hologic, Inc., MA, USA). Partial 16S rRNA gene sequencing allowed differentiation of MAC species. Sequences were analyzed using the MicroSeq system, as described previously (32). Fifty clinical isolates of *M. intracellulare*, including BDQ pretreatment, untreated, and BDQ relapse strains, were chosen for further study. For cases involving BDQ therapy with microbiological relapse, multiple isolates were examined, including at least one pretreatment and one relapse isolate. These isolates are described in Tables 2 and S1.



**Phenotypic susceptibility testing.** MICs to BDQ (0.004  $\mu\text{g/ml}$  to 8  $\mu\text{g/ml}$ ) and CLF (0.06  $\mu\text{g/ml}$  to 2  $\mu\text{g/ml}$ ) were determined using serial two-fold dilutions in cation-adjusted Mueller-Hinton broth plus 5% oleic acid-albumin-dextrose-catalase broth. Because the Clinical and Laboratory Standards Institute (CLSI) has not yet addressed susceptibility testing of *M. intracellulare* with BDQ or CLF, there are no CLSI-recommended breakpoints or established quality control procedures for these drugs. However, our testing procedure is consistent with published recommendations (33). Quality control for CLF was performed with *M. avium* ATCC 700898, as previously recommended (34). Quality control for BDQ was performed using *M. avium* ATCC 700898, and ranges of MICs were recorded in order to assess precision of testing and develop future standards for testing of the drug. Isolates were also tested against clarithromycin (CLR; 0.06  $\mu\text{g/ml}$  to 64  $\mu\text{g/ml}$ ) and amikacin (AMK; 1  $\mu\text{g/ml}$  to 64  $\mu\text{g/ml}$ ) using the CLSI guidelines for clarithromycin and the breakpoints for amikacin suggested by Brown-Elliott et al. (35).

**Whole-genome sequencing and bioinformatic analyses.** Whole-genome sequencing of *M. intracellulare* isolates 1A, 1B, 2A, 2B, and 2C was performed on an Illumina MiSeq platform. Briefly, strains were cultured on Middlebrook 7H11 agar (BD-Canada, Ontario, Canada), and genomic DNA was extracted from a loopful of cells using the MasterPure DNA purification kit (Epicentre, WI, USA). To facilitate lysis, cells were first heated at 80°C for 20 min and then mixed with 600- $\mu\text{m}$  glass beads and vortexed for 5 min. RNA-free genomic DNA was sheared by sonication with a Bioruptor Standard (Diagenode, NJ, USA), and size selection was performed using Agencourt AMPure beads (Beckman Coulter, Ontario, Canada). Paired-end libraries were generated with the NEBNext Ultra DNA library prep kit and NEBNext multiplex oligonucleotides for Illumina (New England Biolabs, Ontario, Canada). A final combined 4 nM library was prepared, denatured, diluted to 14 pM, and sequenced using a MiSeq reagent version 3 kit (600 cycle), according to the manufacturer's guidelines (Illumina, Inc., CA, USA). The SPAdes genome assembler (version 3.6.2) was used for *de novo* generation of contigs (36). Additional analyses, including reference-based mapping of sequencing reads to contigs and identification of high-quality single nucleotide variants (SNVs), were performed with the Geneious R7 package (Biomatters, Auckland, New Zealand) (37). MEGA5 was used for phylogenetic analyses (38).

**Targeted gene sequencing.** DNA sequences of the *mmpT5* and *atpE* genes were determined by targeted gene sequencing. *M. intracellulare* strains were cultured on agar medium, and genomic DNA was extracted using the PrepMan Ultra reagent (Life Technologies, CA, USA). Briefly, a loopful of bacteria was suspended in 100  $\mu\text{l}$  of preparation reagent. Samples were held for 30 s, heated for 10 min at 100°C, allowed to cool at room temperature (20 to 22°C) for 2 min, and then centrifuged at maximum speed in a microcentrifuge for 2 min. The DNA-containing supernatant (50  $\mu\text{l}$ ) was retained for PCR and sequencing.

PCR amplification of the *mmpT5* gene was performed in a 20- $\mu\text{l}$  reaction using 1 $\times$  FailSafe Premix I, 1.25 U of FailSafe enzyme mix (Epicentre, WI, USA), and 10  $\mu\text{M}$  each primer (*mmpT5\_F*, 5'-GATGGCA CCTTTGACTGC-3'; *mmpT5\_R*, 5'-GCTGGTGTTCAGTCACTTC-3'). The PCR conditions included an initial denaturation of 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing of 54°C for 1 min, and elongation at 72°C for 1 min, with a final elongation at 72°C for 5 min.

PCR amplification of the *atpE* gene was performed in a 20- $\mu\text{l}$  reaction using 1 $\times$  FailSafe Premix I, 1.25 U of FailSafe enzyme mix (Epicentre, WI, USA), and a 10  $\mu\text{M}$  concentration of each primer (*atpE\_F*, 5'-CCCTACCAGATATCAAGGAGGATAAG-3'; *atpE\_R*, 5'-CACCCATCACAGCGAACTAG-3'). The *atpE*-specific PCR conditions included an initial denaturation of 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing of 54°C for 30 s, and elongation at 72°C for 30 s, plus a final elongation at 72°C for 7 min.

Agarose gel electrophoresis was used to check that amplicons had been generated and were of the correct size (615 bp for *mmpT5* and 298 bp for *atpE*). In preparation for sequencing, 8  $\mu\text{l}$  of PCR product was combined with 2  $\mu\text{l}$  of ExoSAP-IT reagent (Affymetrix, CA, USA). Sequencing was performed using the BigDye Terminator version 3.1 cycle sequencing kit and the *mmpT5\_F/mmpT5\_R* or *atpE\_F/atpE\_R* primer pairs on an ABI 3500 genetic analyzer (Thermo Fisher Scientific, CA, USA). DNA sequence analysis was performed using RipSeq software (Pathogenomix, CA, USA). Phylogenetic analysis was performed using MEGA5 (38). The genome of *M. intracellulare* ATCC 13950<sup>T</sup> (GenBank accession no. GCA\_000172115.1) was designated the wild type.

**Accession number(s).** Data associated with this study have been registered as BioProject ID PRJNA339272. Raw sequencing data from *M. intracellulare* isolates 1A, 1B, 2A, 2B, and 2C have been deposited in the Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra>) as BioSample SAMN05770403 through SAMN05770407. Nucleotide sequences of 17 representative *mmpT5* alleles have been deposited in GenBank as accession numbers KX826800 through KX826816. Additional *mmpT5* sequences were derived from the following publicly available data sets: *M. intracellulare* ATCC 13950<sup>T</sup> (GenBank accession no. GCA\_000172115.1), *M. intracellulare* GMI 1956 (GenBank accession no. GCA\_000523815.1), *M. intracellulare* GMI 1280 (GenBank accession no. GCA\_000524015.1), *M. intracellulare* M.i.198 (GenBank accession no. GCA\_000309055.1), *M. intracellulare* MOTT-02 (GenBank accession no. GCA\_000277145.1), *M. yongonense* 05-1390<sup>T</sup> (accession no. NC\_021715.1), *M. paraintracellulare* MOTT-64<sup>T</sup> (accession no. NC\_016948.1), *M. chimaera* MCIMRL2 (GenBank accession no. GCA\_001307335.1), *Mycobacterium* sp. strain H4Y (GenBank accession no. GCA\_000364405.1), *Mycobacterium* sp. MOTT36Y (GenBank accession no. GCA\_000262165.1), *M. colombiense* CECT 3035<sup>T</sup> (GenBank accession no. GCA\_000222105.4), *M. avium* subsp. *avium* ATCC 25291<sup>T</sup> (GenBank accession no. GCA\_000174035.1), *M. avium* subsp. *silvaticum* ATCC 49884<sup>T</sup> (GenBank accession no. GCA\_000504975.1), *M. avium* subsp. *paratuberculosis* ATCC 19698<sup>T</sup> (GenBank accession no. GCA\_000240525.2), and "*M. avium* subsp. *hominissuis*" 104 (accession no. NC\_008595.1).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.02087-16>.

**TEXT S1**, PDF file, 0.1 MB.

**TEXT S2**, PDF file, 0.06 MB.

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