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Article

Dual Mutations in *MSMEG_0965* and *MSMEG_1380* Confer High-Level Resistance to Bortezomib and Linezolid by both Reducing Drug Intake and Increasing Efflux in *Mycobacterium smegmatis*

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Abstract: The emergence of multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* strains poses serious challenges to global tuberculosis control, highlighting the urgent need to unravel the mechanisms underlying multidrug resistance. In this study, we screened for spontaneous bortezomib (BTZ)-resistant mutants of *Mycobacterium smegmatis* and identified a strain, Msm-R1-2, which exhibited high-level resistance to both BTZ and linezolid. Whole-genome sequencing revealed mutations in *MSMEG_1380* and *MSMEG_0965* genes, encoding a transcriptional regulator (involved in regulating efflux pump expression) and a porin, respectively are potential contributors to drug resistance. CRISPR-Cpf1-assisted gene knockout and editing experiments confirmed that dual mutations in *MSMEG_1380* and *MSMEG_0965* synergistically enhanced resistance to BTZ and LZD, conferring cross-resistance to other antibiotics, including moxifloxacin and clofazimine. Ethidium bromide accumulation assay demonstrated that mutations in *MSMEG_0965* reduce cell wall permeability, contributing to multidrug resistance. Furthermore, previous studies have shown that mutations in *MSMEG_1380* upregulate the *mmpS5-mmpL5* efflux system, thereby promoting drug efflux and reducing intracellular drug concentrations, while mutations in *MSMEG_0965* impair porin function, limiting antibiotic uptake and significantly contributing to the multidrug-resistant phenotype. Collectively, these findings provide valuable insights into the molecular mechanisms of mycobacterial drug resistance, underscoring the pivotal roles of efflux and uptake pathways in the development of multidrug resistance.

Keywords: *Mycobacterium smegmatis*; bortezomib; linezolid; drug resistance mechanism

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains a major global health challenge, particularly with the emergence of multidrug-resistant (MDR) and extensively drug-

resistant (XDR) strains [1,2]. These resistant strains severely compromise current treatment regimens, underscoring the urgent need for novel therapeutic strategies [2,3]. Among the promising candidates, bortezomib (BTZ), a proteasome inhibitor originally approved for the treatment of multiple myeloma, has shown potent activity against Mtb [4]. Linezolid (LZD), an oxazolidinone antibiotic, is already an established component of TB treatment regimens [1,5]. However, the mechanisms underlying BTZ resistance remain poorly understood [6], and while LZD resistance has been primarily attributed to ribosomal mutations, non-ribosomal resistance mechanisms remain largely unexplored [7]. Thus, elucidating these resistance mechanisms is crucial for developing more effective therapeutic strategies.

Previous studies suggest that BTZ inhibits Mtb proteolysis by targeting ClpP1P2, a caseinolytic protease complex encoded by *clpP1* and *clpP2* [8–10], and may also act on the Mtb proteasome complex PrcBA, encoded by *prcB* and *prcA* [11,12]. LZD, on the other hand, inhibits protein synthesis by binding to the 50S ribosomal subunit [13,14]. However, some resistant strains lack mutations in these established targets, suggesting the involvement of alternative resistance pathways, like MmpL9, and EmbB-EmbC [15–17]. Efflux pump systems, such as the MmpS5-MmpL5 transporter, have been implicated in conferring drug resistance in mycobacteria by actively extruding antimicrobial compounds [18,19]. In *Mycobacterium smegmatis* (Msm), mutations in *MSMEG_1380*, encoding a TetR-family transcriptional regulator, have been shown to upregulate efflux pump expression, conferring resistance to multiple drugs, including clofazimine (CFZ) and bedaquiline (BDQ) [20,21]. In addition, porins such as *MSMEG_0965* (MspA) play a critical role in drug uptake by facilitating the transport of hydrophilic molecules across the mycobacterial cell wall [22,23]. Mutations in genes encoding porins can reduce membrane permeability, thereby diminishing the efficacy of antibiotics such as fluoroquinolones and potentially leading to treatment failure or prolonged therapy duration [24]. While these resistance mechanisms have been individually characterized, their potential interplay in mediating resistance, particularly in strains with multiple mutations, remains unclear [25].

In this study, we used the nonpathogenic Msm mc²155 as a model organism [26] to investigate drug resistance mechanisms in mycobacteria. By screening spontaneous BTZ-resistant mutants and performing whole-genome sequencing (WGS), we identified multiple resistance-associated mutations, despite the absence of mutations in the known BTZ target, suggesting the involvement of alternative resistance mechanisms. Notably, among these mutants, we identified a strain exhibiting high-level resistance to both BTZ and LZD. CRISPR-Cpf1-assisted gene knockout, gene editing, overexpression, and complementation experiments revealed that *MSMEG_1380* and *MSMEG_0965* are key determinants of resistance. Single-gene mutations conferred low-level resistance, while double-gene mutations significantly enhanced resistance to BTZ, LZD, and other antibiotics. The ethidium bromide (EtBr) accumulation assay demonstrated that mutations in *MSMEG_0965* led to a significant reduction in cell wall permeability, resulting in lower intracellular drug concentrations. Furthermore, previous studies have shown that mutations in *MSMEG_1380* promote drug efflux through the upregulation of MmpS5-MmpL5 system [27,28]. These findings highlight the synergistic role of *MSMEG_1380* and *MSMEG_0965* in conferring high-level resistance, emphasizing the multifactorial mechanisms between drug uptake and efflux mechanisms. This study advances our understanding of mycobacterial drug resistance and provides valuable insights into potential therapeutic targets for combating MDR/XDR strains.

2. Results

2.1. High Frequency of Spontaneous Mutations Conferring BTZ Resistance in Msm

To investigate potential BTZ targets, we screened Msm mc²155 strains for spontaneous resistance to BTZ. First, we determined that the minimum inhibitory concentration (MIC) of BTZ for Msm was 5 µg/mL. Msm strains were then plated on 7H10 agar plates containing increasing concentrations of BTZ (20 × MIC, 40 × MIC, and 60 × MIC) in four independent batches. From each batch, 10–20 single colonies were randomly selected from each batch, yielding a total of 57 resistant colonies. Subsequently, these colonies were cultured, and the MIC was determined to assess the resistance

levels. All strains exhibited MIC ≥ 20 $\mu\text{g/mL}$ (listed in Table S1), with a spontaneous mutation rate ranging from 1.86×10^{-7} to 6.60×10^{-7} . In contrast, previous studies reported a spontaneous mutation rate of only 6×10^{-10} in Mtb H37Rv strains screened for rifampin (RIF) resistance on 7H10 agar containing 5 or 10 $\mu\text{g/mL}$ RIF [29], which is three orders of magnitude lower than the rate observed for BTZ-resistant strains. These findings indicate a relatively high mutation rate in BTZ-resistant Msm strains.

2.2. WGS of BTZ-Resistant Msm Strains Revealed a Variety of Mutations across Different Genes

Sanger sequencing of *prcA*, *prcB*, and *clpP1P2*, which encode known BTZ targets, revealed no mutations in the resistant strains. To further evaluate their roles in BTZ susceptibility, we constructed Msm strains with knockouts of *prcA* or *prcB*, overexpression of *clpP1P2*, and knockdowns of *clpP1* or *clpP2*. The results showed no significant correlation was found between BTZ susceptibility and these genetic modifications (Figure S1), suggesting that BTZ resistance in Msm may involve alternative resistance mechanisms beyond these known targets.

To further explore potential resistance mechanisms, we randomly selected 5 resistant strains from the 57 isolates and parent strain for WGS. The sequencing analysis identified 35 unique mutations across various genes (Table S2). Among them, *MSMEG_3244* was the most frequently mutated gene (4/5 strains), followed by *MSMEG_5085* and *MSMEG_3987* (2/5 strains). Mutations in *MSMEG_1380* and *MSMEG_0965* were detected in only one strain. Sanger sequencing of these genes across all 57 isolates revealed mutation frequencies of 7% (*MSMEG_3244*), 42% (*MSMEG_5085*), 17% (*MSMEG_3987*), 3% (*MSMEG_1380*), and 3% (*MSMEG_0965*). Further validation is required to determine the association of these genes with BTZ resistance.

2.3. BTZ-Resistant Msm Strains Exhibited Cross-Resistance to Other Antibiotics

To determine whether the spontaneous drug-resistant mutants exhibited cross-resistance, we tested the susceptibility of WGS-analyzed strains to a range of antibiotics (Table 1). Most resistant strains showed no significant changes (a 4-fold change in MIC) in their sensitivity to antibiotics such as levofloxacin (LEV), amikacin (AMK), and streptomycin (STR). However, some strains exhibited notable cross-resistance. For instance, Msm-R1-2 demonstrated high resistance to both BTZ and LZD, with MICs rising to 80 $\mu\text{g/mL}$ and 128 $\mu\text{g/mL}$, respectively. Additionally, the MIC of clarithromycin (CLR) increased 4-fold. Similarly, Msm-R1-13 exhibited an 8-fold increase in MIC of CLR and a 4-fold increase in MIC of gentamicin (GEN), while Msm-R4-1 showed a 4-fold increase in MIC of ethambutol (EMB). These observations suggest that resistance mechanisms may involve shared pathways that regulate cross-resistance, warranting further investigation.

Table 1. MICs of various antibiotics against BTZ-resistant Msm strains.

Strains	MICs ($\mu\text{g/mL}$)							
	BTZ	LZD	CLR	EMB	GEN	LEV	AMK	STR
Msm	5	2	1	1	2	0.25	0.5	0.5
Msm-R1-2 [#]	80	128	4	2	2	0.25	0.5	0.25
Msm-R1-13 [#]	>80	4	8	1	8	0.25	0.5	0.25
Msm-R3-2 [#]	80	4	2	2	4	0.25	0.5	1
Msm-R4-1 [#]	80	2	2	4	1	0.5	0.5	0.5
Msm-R4-7 [#]	80	2	1	1	1	0.5	1	0.5

Msm: wide type Msm strain; [#]BTZ-resistant Msm strains. BTZ, borte-zomib; LZD, linezolid; CLR, clarithromycin; EMB, ethambutol; GEN, gentamicin; LEV, levofloxacin; AMK, amikacin; STR, streptomycin.

2.4. Single Knockout of MSMEG_1380 or MSMEG_0965 in Msm Resulted in Low-Level Resistance to BTZ and LZD

To further investigate the genes associated with Msm's resistance to BTZ, we used CRISPR-Cpf1-assisted recombineering technology [30] to knock out *MSMEG_3244*, *MSMEG_5085*, *MSMEG_3987*,

MSMEG_1380, and *MSMEG_0965* genes. We then tested the MICs of BTZ against these knockout strains. The results showed that compared to Msm, Msm^{Δ3244}, Msm^{Δ5085}, and Msm^{Δ3987} exhibited no significant changes in resistance to BTZ, indicating that these genes are not involved in the resistance mechanism. In contrast, Msm^{Δ1380} and Msm^{Δ0965} resulted in a 4-fold increase in the MIC of BTZ compared to Msm, suggesting that *MSMEG_1380* and *MSMEG_0965* play crucial roles in Msm's resistance to BTZ (Table 2).

Table 2. MICs of BTZ against Msm, Msm^{Δ3244}, Msm^{Δ5085}, Msm^{Δ3987}, Msm^{Δ1380}, and Msm^{Δ0965}.

Strains	BTZ MIC (μg/mL)
Msm	5
Msm ^{Δ3244}	5
Msm ^{Δ5085}	5
Msm ^{Δ3987}	5
Msm ^{Δ1380}	20
Msm ^{Δ0965}	20

Msm: wide type Msm strain; Msm^{Δ3244}, *MSMEG_3244* knockout Msm strain; Msm^{Δ5085}, *MSMEG_5085* knockout Msm strain; Msm^{Δ3987}, *MSMEG_3987* knockout Msm strain; Msm^{Δ1380}, *MSMEG_1380* knockout Msm strain; Msm^{Δ0965}, *MSMEG_0965* knockout Msm strain. BTZ, bortezomib.

Given that Msm-R1-2, a strain exhibiting high-level resistance to both BTZ and LZD, harbors mutations in *MSMEG_1380* and *MSMEG_0965* (Tables 1 and S2), we further evaluated the LZD susceptibility of Msm^{Δ1380} and Msm^{Δ0965} to determine their individual contributions to resistance. The results showed that, compared to the wild-type Msm, both Msm^{Δ1380} and Msm^{Δ0965} exhibited a 4-fold increase in the MIC of LZD (Table 3). These findings suggest that *MSMEG_1380* and *MSMEG_0965* are key contributors to BTZ and LZD resistance in Msm.

Table 3. MICs of LZD against Msm, Msm^{Δ1380}, and Msm^{Δ0965}.

Strains	LZD MIC (μg/mL)
Msm	2
Msm ^{Δ1380}	8
Msm ^{Δ0965}	8

Msm: wide type Msm strain; Msm^{Δ1380}, *MSMEG_1380* knockout Msm strain; Msm^{Δ0965}, *MSMEG_0965* knockout Msm strain. LZD, linezolid.

2.5. Single Knockout of *MSMEG_1380* or *MSMEG_0965* in Msm Affected the Sensitivity of to Other Antibiotics

Previous studies have suggested that *MSMEG_1380* is associated with drug efflux mechanisms, while *MSMEG_0965* is linked to drug uptake processes[28,31]. Mutations in these genes are known to confer resistance to multiple antibiotics. To investigate the roles of *MSMEG_1380* and *MSMEG_0965*, we tested the susceptibility of Msm^{Δ1380} and Msm^{Δ0965} to a range of antibiotics. Both knockout strains (Msm^{Δ1380} and Msm^{Δ0965}) exhibited resistance to CFZ, moxifloxacin (MOX), and LEV. Interestingly, BDQ resistance was observed only in Msm^{Δ0965}, indicating that *MSMEG_0965* plays a specific role in mediating BDQ resistance (Figure 1). In addition, both Msm^{Δ1380} and Msm^{Δ0965} displayed a 4-fold increase in MIC of vancomycin (VAN). Furthermore, Msm^{Δ0965} also exhibited an 8-fold increase in MIC of CLR, a 4-fold increase in MIC of sulfadiazine (SDZ), and a 16-fold increase in MIC of sulfamethoxazole (SMX) (Table 4). In contrast, both knockout strains (Msm^{Δ1380} and Msm^{Δ0965}) showed no significant differences in MICs for EMB, STR, GEN, and AMK compared to the wild type Msm (Table S3). These findings suggest that while the knockout of *MSMEG_1380* or *MSMEG_0965*

in Msm strains affects sensitivity to various antibiotics, with distinct and gene-specific mechanisms of action that do not apply universally to all antibiotics, such as BDQ.

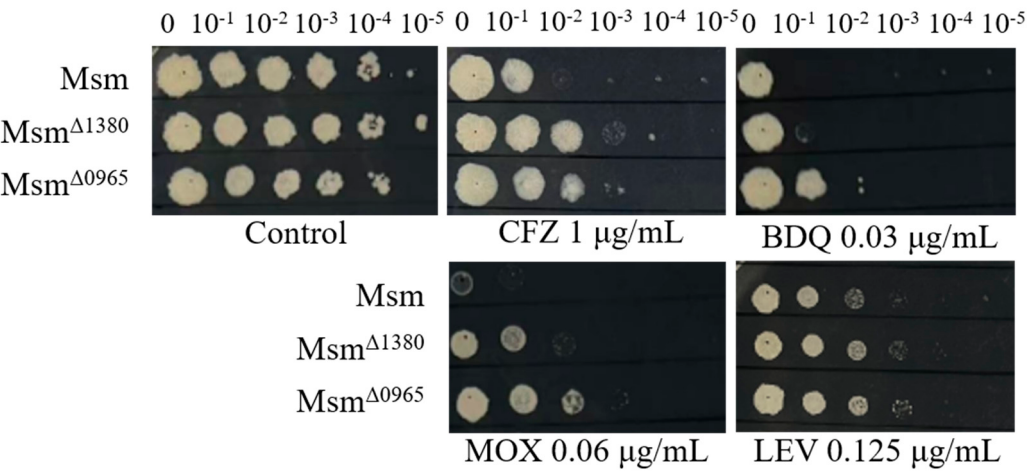


Figure 1. The sensitivity of Msm, Msm^{Δ1380}, and Msm^{Δ0965} to various antibiotics. Msm: wide type Msm strain; Msm^{Δ1380}, *MSMEG_1380* knockout Msm strain; Msm^{Δ0965}, *MSMEG_0965* knockout Msm strain. CFZ, clofazimine; BDQ, bedaquiline; MOX, moxifloxacin; LEV, levofloxacin.

Table 4. MICs of multi-drugs against Msm, Msm^{Δ1380}, and Msm^{Δ0965}.

Strains	MICs (μg/mL)			
	VAN	CLR	SDZ	SMX
Msm	8	2	2	1
Msm ^{Δ1380}	32	2	2	1
Msm ^{Δ0965}	32	16	8	16

Msm: wide type Msm strain; Msm^{Δ1380}, *MSMEG_1380* knockout Msm strain; Msm^{Δ0965}, *MSMEG_0965* knockout Msm strain. VAN, vancomycin; CLR, clarithromycin; SDZ, sulfadiazine; SMX, sulfamethoxazole.

2.6. Overexpression of *MSMEG_1380* or *MSMEG_0965* in Msm and Complementation of these Genes in Knockout Strains Affected the Drug Sensitivity to BTZ and LZD

To further investigate the functional contributions of *MSMEG_1380* and *MSMEG_0965* to drug susceptibility phenotypes, we generated Msm recombinant strains harbouring overexpression constructs for these genes and performed genetic complementation in their respective knockout backgrounds. Overexpression and genetic complementation of *MSMEG_1380* were placed under the transcriptional control of the *hsp60* promoter, while the corresponding manipulations for *MSMEG_0965* utilised its native promoter. Drug susceptibility testing (DST) revealed that Msm::1380 and Msm::0965 strains displayed enhanced sensitivity to BTZ and LZD. Genetic complementation of Msm^{Δ1380::C1380} partially restored sensitivity to BTZ, LZD, CFZ. Similarly, complementation of Msm^{Δ0965::C0965} wild-type susceptibility levels to BTZ, LZD, MOX, and CFZ. Notably, BDQ susceptibility remained unaltered in the Msm^{Δ0965::C0965} strain, suggesting a gene-specific interaction between *MSMEG_0965* and intrinsic BDQ resistance mechanisms (Figure 2). We also evaluated the susceptibility of these strains to other antibiotics, including VAN and CLR. The MICs of these antibiotics in the overexpression strains remained equivalent to those of wild-type Msm, and the MICs in the complementation strains showed no significant changes compared to their respective knockout strains (Table S4). These results suggest that overexpression of *MSMEG_1380* or *MSMEG_0965*, and complementation in the respective knockout strains affect specific drug

sensitivity, but their underlying regulatory mechanisms differ, highlighting the complexity of the regulatory networks involved.

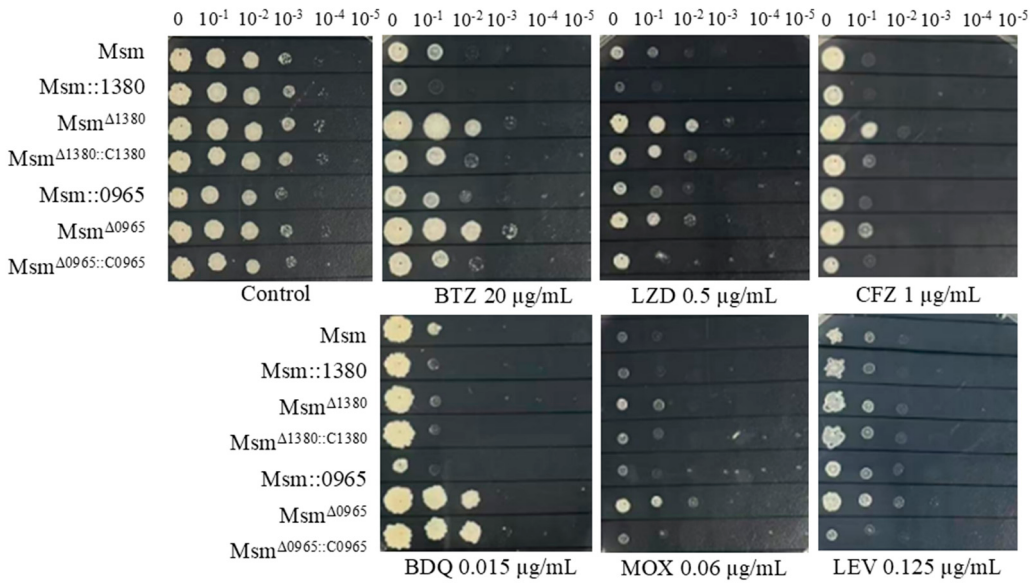


Figure 2. The sensitivity of recombinant strains to various antibiotics. Msm: wide type Msm strain; Msm::1380, *MSMEG_1380* overexpression Msm strain; Msm Δ 1380, *MSMEG_1380* knockout Msm strain; Msm Δ 1380::C1380, *MSMEG_1380* complemented Msm Δ 1380 strain; Msm::0965, *MSMEG_0965* overexpression Msm strain; Msm Δ 0965, *MSMEG_0965* knockout Msm strain; Msm Δ 0965::C0965, *MSMEG_0965* complemented Msm Δ 0965 strain. BTZ, bortezomib; LZD, linezolid; CFZ, clofazimine; BDQ, bedaquiline; MOX, moxifloxacin; LEV, levofloxacin.

2.7. Roles of *MSMEG_1380*^{12insC} and *MSMEG_0965*^{400insCC} in Multidrug Resistance were Confirmed by Gene Editing in Msm

To verify that single mutations in *MSMEG_1380* or *MSMEG_0965* confer resistance to multiple drugs, we employed CRISPR-Cpf1-assisted recombineering technology [30] to edit the respective mutation sites in these genes. The drug susceptibility profiles of the single gene-edited strains were consistent with those of Msm Δ 1380 and Msm Δ 0965 (Figure 1, Tables 4 and 5, Figure 3). These results indicated that single knockout or mutation of *MSMEG_1380* or *MSMEG_0965* in Msm conferred only low-level resistance to BTZ and LZD rather than the high-level resistance observed in the Msm-R1-2 strain. These findings confirm the important roles of *MSMEG_1380* and *MSMEG_0965* in conferring drug resistance in Msm. However, the precise mechanisms underlying the high-level resistance to BTZ and LZD observed in the Msm-R1-2 remain to be further elucidated.

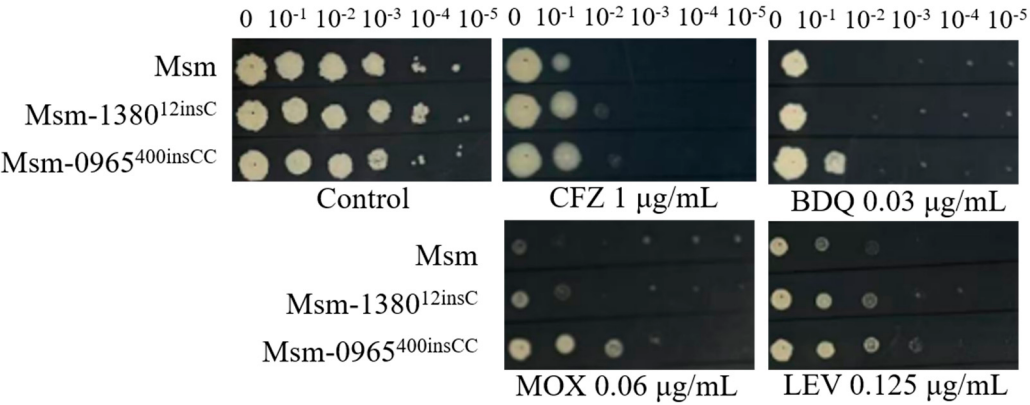


Figure 3. The sensitivity of single gene-edited strains to various antibiotics. Msm: wide type Msm strain; Msm-1380^{12insC}, Msm strain with a 12insC insertion mutation in *MSMEG_1380*; Msm-0965^{400insCC}, Msm strain with a

400insCC insertion mutation in *MSMEG_0965*. CFZ, clofazimine; BDQ, bedaquiline; MOX, moxifloxacin; LEV, levofloxacin.

Table 5. MICs of multi-drugs against single gene-edited strains.

Strains	MICs (μg/mL)					
	BTZ	LZD	VAN	CLR	SDZ	SMX
Msm	5	2	8	2	2	1
Msm-1380 ^{e12insC}	20	8	32	2	2	1
Msm-0965 ^{e400insCC}	20	8	32	16	8	16

Msm: wide type Msm strain; Msm-1380^{e12insC}, Msm strain with a 12insC insertion mutation in *MSMEG_1380*; Msm-0965^{e400insCC}, Msm strain with a 400insCC insertion mutation in *MSMEG_0965*. BTZ, borteomib; LZD, linezolid; VAN, vancomycin; CLR, clarithromycin; SDZ, sulfadiazine; SMX, sulfamethoxazole.

2.8. Dual Mutations of *MSMEG_1380* and *MSMEG_0965* in Msm Exhibited High-Level Resistance to BTZ and LZD

Based on the above results, we hypothesize that the dual mutations in *MSMEG_1380* and *MSMEG_0965* are the primary cause of high-level resistance in Msm [32,33]. To validate this hypothesis, we used CRISPR-Cpf1-assisted recombineering technology [30] to perform the dual knockouts and gene editing strains of these two genes in Msm. DST revealed that the MICs of BTZ and LZD against the dual mutation strains (Msm^{Δ1380Δ0965} and Msm-1380^{e12insC}-0965^{e400insCC}) were 16-fold and 64-fold higher respectively than those to Msm (Table 6), which was consistent with the MIC values observed in Msm-R1-2 (Table 1). Compared to the single-gene mutant strains, the dual mutation strains also exhibited higher resistance to MOX and LEV (Figures 1, 3, and 4), and increased resistance to VAN (Tables 4–6). The resistance levels of the double mutation strains to CLR, SDZ, and SMX were comparable to those of Msm^{Δ0965} and Msm-0965^{e400insCC} (Tables 4–6). Interestingly, the complementation of *MSMEG_1380* and *MSMEG_0965* in the double-knockout strains did not alter the MICs of multi-drugs (Table 6). Based on the MIC results of the dual knockout and dual gene-edited strains, we propose that the dual mutations of *MSMEG_1380* and *MSMEG_0965* in Msm are the main reason for Msm's high-level resistance to BTZ and LZD, and they also influence the sensitivity to other antibiotics.

Table 6. MICs of multi-drugs against single gene-edited strains.

Strains	MICs (μg/mL)					
	BTZ	LZD	VAN	CLR	SDZ	SMX
Msm	5	2	8	2	2	1
Msm ^{Δ1380Δ0965}	80	128	128	16	16	16
Msm-1380 ^{e12insC} -0965 ^{e400insCC}	80	128	128	16	16	16
Msm ^{Δ1380Δ0965::C1380C0965}	80	128	128	16	16	16

Msm: wide type Msm strain; Msm^{Δ1380Δ0965}, *MSMEG_1380* and *MSMEG_0965* double knockout Msm strain; Msm^{Δ1380Δ0965::C1380C0965}, *MSMEG_1380* and *MSMEG_0965* double complemented Msm^{Δ1380Δ0965} strain; Msm-1380^{e12insC}-0965^{e400insCC}, Msm strain with a 12insC insertion in *MSMEG_1380* and a 400insCC insertion in *MSMEG_0965*. BTZ, borteomib; LZD, linezolid; VAN, vancomycin; CLR, clarithromycin; SDZ, sulfadiazine; SMX, sulfamethoxazole.

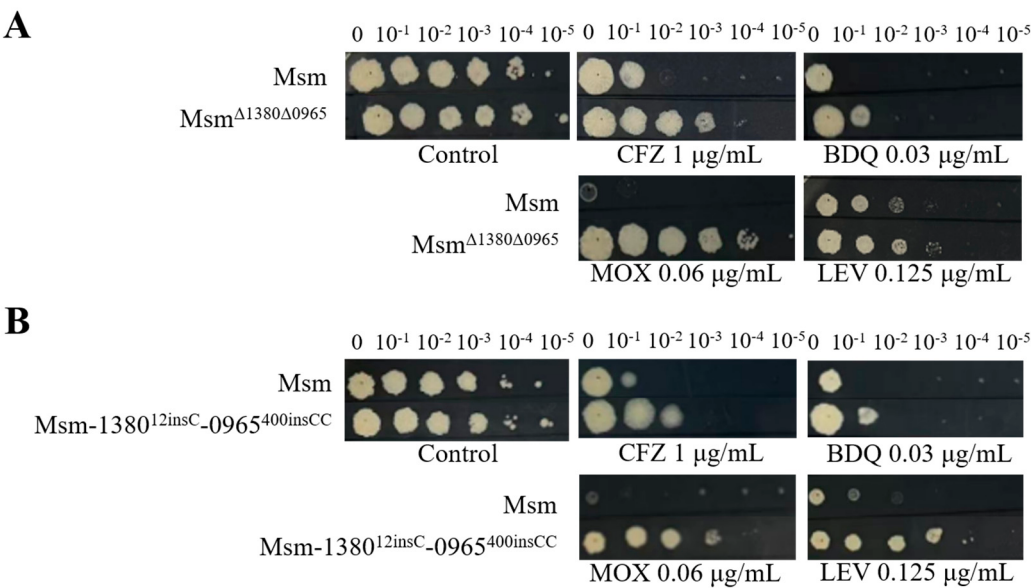


Figure 4. Dual mutations of *MSMEG_1380* and *MSMEG_0965* in *Msm* affect *Msm* sensitivity to various antibiotics. (A) The sensitivity of *Msm* and *Msm*^{Δ1380Δ0965} to various antibiotics. (B) The sensitivity of *Msm* and *Msm*-1380^{e12insC}-0965^{e400insCC} to various antibiotics. *Msm*: wide type *Msm* strain; *Msm*^{Δ1380Δ0965}, *MSMEG_1380* and *MSMEG_0965* double knockout *Msm* strain; *Msm*-1380^{e12insC}-0965^{e400insCC}, *Msm* strain with a 12insC insertion in *MSMEG_1380* and a 400insCC insertion in *MSMEG_0965*. CFZ, clofazimine; BDQ, bedaquiline; MOX, moxifloxacin; LEV, levofloxacin.

2.8. Knockout and Mutation of *MSMEG_0965* Reduced Cell Wall Permeability

Based on the function of the protein encoded by *MSMEG_0965* [23], we hypothesized that mutations in *MSMEG_0965* might impair cell wall permeability [34,35]. To test this hypothesis, we performed the EtBr accumulation assay[36] to evaluate cell wall permeability in *Msm*-R1-2, knockout and gene-edited *Msm* strains. The results showed that *Msm*-R1-2, *Msm*^{Δ0965} and *Msm*-0965^{e400insCC} strains exhibited significantly reduced cell wall permeability, while the permeability of *Msm*^{Δ1380} and *Msm*-1380^{e12insC} remained unchanged. Similarly, the permeability of *Msm*^{Δ1380Δ0965} and *Msm*-1380^{e12insC}-0965^{e400insCC} was also significantly decreased (Figure 5). These results indicate that knockout and mutation of *MSMEG_0965* reduce cell wall permeability, thereby contributing to drug resistance, while *MSMEG_1380* does not appear to affect cell wall permeability.

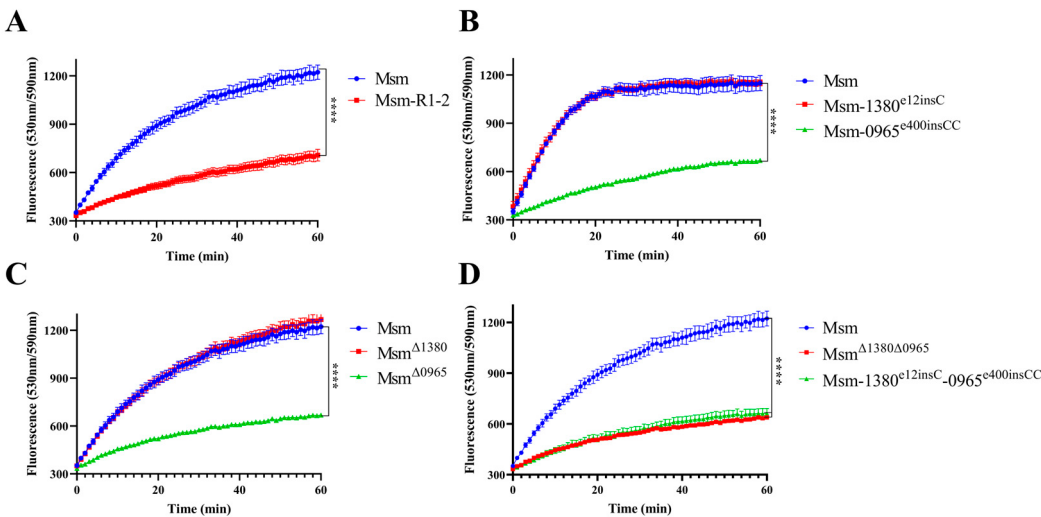


Figure 5. *MSMEG_0965* affects cell wall permeability in *Msm*. (A) EtBr accumulation in *Msm* and *Msm*-R1-2. (B) EtBr accumulation in single knockout strains. (C) EtBr accumulation in single gene-edited strains. (D) EtBr accumulation in double knockout and double gene-edited strains. ****, $P < 0.0001$. Data are presented as mean $\pm$ standard deviation, $n=3$. *Msm*: wide type *Msm* strain; *Msm*-R1-2, BTZ-resistant strains; *Msm* Δ^{1380} , *MSMEG_1380* knockout *Msm* strain; *Msm* Δ^{0965} , *MSMEG_0965* knockout *Msm* strain; *Msm*-1380^{e12insC}, *Msm* strain with a 12insC insertion mutation in *MSMEG_1380*; *Msm*-0965^{e400insCC}, *Msm* strain with a 400insCC insertion mutation in *MSMEG_0965*; *Msm* $\Delta^{1380\Delta 0965}$, *MSMEG_1380* and *MSMEG_0965* double knockout *Msm* strain; *Msm*-1380^{e12insC}-0965^{e400insCC}, *Msm* strain with a 12insC insertion in *MSMEG_1380* and a 400insCC insertion in *MSMEG_0965*.

3. Discussion

The emergence of MDR and XDR *Mtb* strains presents a critical barrier to global TB control, emphasizing the urgent need for new therapeutic strategies [1,37]. This study provides novel insights into the mechanisms underlying BTZ and LZD resistance, identifying double mutations in *MSMEG_1380* and *MSMEG_0965* as key contributors to high-level resistance. These findings highlight the synergistic effect of efflux and uptake pathways in mediating drug resistance. This new and previously unreported phenomenon underscores the importance of multifactorial resistance mechanisms in mycobacteria.

DST revealed that certain BTZ-resistant strains, such as *Msm*-R1-2, exhibit cross-resistance to LZD and CLR, suggesting a network effect where BTZ resistance influences the activity of other drugs [38–40]. Notably, single-gene mutations in *MSMEG_1380* or *MSMEG_0965* result in low-level resistance to BTZ and LZD (4-fold increase in MIC), while double mutations produce a synergistic effect, raising MICs by 16-fold and 64-fold, respectively. Interestingly, resistance levels to CLR, SDZ, and SMX in the double-mutant strain aligned with those observed in the *MSMEG_0965* single mutant strain, suggesting *MSMEG_0965* primarily mediates resistance to these drugs (Tables 3–5).

Further characterization of single-gene knockout strains revealed that *Msm* Δ^{0965} , but not *Msm* Δ^{1380} (Figure 1), confers resistance to BDQ [41,42]. This finding suggests a lack of clear genotype-phenotype correlation for BDQ resistance in this context and implicates MspA porin may be a critical determinant [22,43–45]. Complementation and overexpression experiments showed that *MSMEG_1380* and *MSMEG_0965* influence drug sensitivity to BTZ, LZD, and some other antibiotics, but their effects on drugs like LEV, VAN, and CLR remained unchanged. These results highlight the potential strain-dependent effects of genetic modifications and the complexity of antibiotic resistance regulation [46].

Mechanistically, *MSMEG_1380* and *MSMEG_0965* appear to act synergistically to confer high-level resistance to BTZ and LZD. *MSMEG_1380* likely enhances the expression or activity of the MmpS5-MmpL5 system, promoting increased drug efflux and reduced intracellular drug concentrations [27,28]. In parallel, *MSMEG_0965* modulates the MspA porin, altering cell wall permeability and limiting the passive diffusion of antibiotics [22,43,47]. Together, these dual mechanisms significantly contribute to the observed multidrug resistance phenotype, highlighting the complex regulatory interplay between efflux and uptake systems [32,33,48] (Figure 6). Our findings provide phenotypic evidence for the synergistic action of *MSMEG_1380* and *MSMEG_0965*, supporting a multifactorial regulatory model of resistance [38–40].

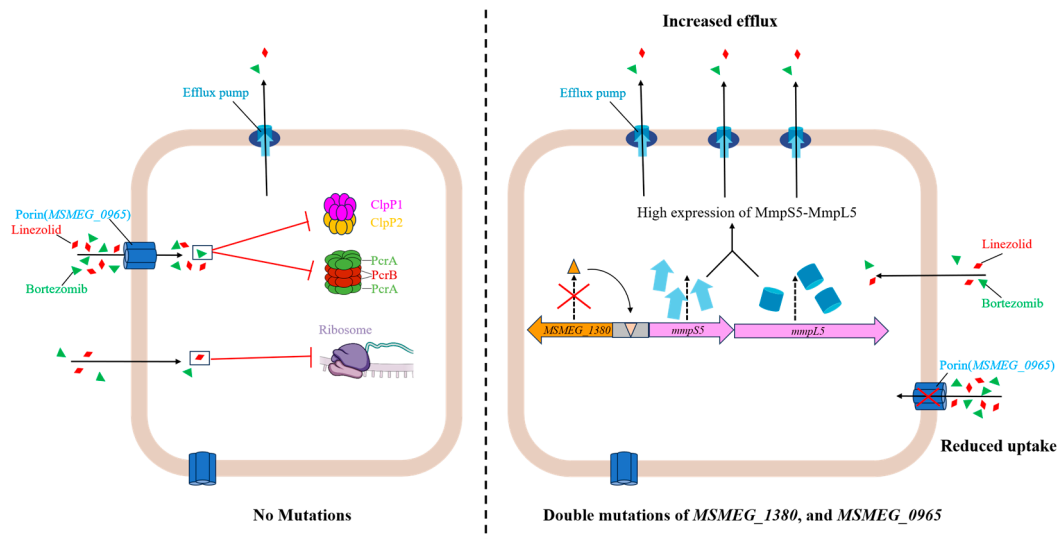


Figure 6. Schematic representation of the mechanism underlying high-level resistance to BTZ and LZD in Msm harboring mutations in *MSMEG_1380* and *MSMEG_0965*.

Despite these advances, the study has limitations. First, these findings were observed in Msm, a non-pathogenic model organism, and their relevance to clinical Mtb strains requires further validation. Second, while the phenotypic effects of *MSMEG_1380* and *MSMEG_0965* mutations are well-characterized, their precise molecular mechanisms are not yet fully understood. Functional studies, including protein structure-function analyses and investigations of regulatory pathways, are essential to further elucidate their roles. Lastly, other potential resistance pathways and mutations were not explored in this study, warranting further investigation.

Future studies should focus on validating these findings in Mtb clinical isolates, elucidating the molecular mechanisms of *MSMEG_1380* and *MSMEG_0965*, and identifying additional resistance-associated pathways. Therapeutic strategies targeting the MmpS5-MmpL5 efflux system or restoring porin functionality could provide promising avenues for overcoming resistance. Furthermore, continued exploration of BTZ resistance mechanisms will be critical for gaining valuable insights and providing the base for the development of next-generation anti-TB therapies.

4. Materials and Methods

4.1. Strains, Plasmids, and Culture Conditions

All mycobacterial strains were cultured at 37°C in 7H9 liquid medium supplemented with 0.05% Tween 80, 0.2% glycerol, and 10% OADC, or on 7H10/7H11 solid medium. Antibiotics were added to the media as required: 50 µg/mL kanamycin (KAN) and 30 µg/mL zeocin (ZEO). For induced expression, 200 ng/mL anhydrotetracycline (aTc) was added. All *Escherichia coli* strains were cultured in LB medium supplemented with 50 µg/mL KAN, 30 µg/mL ZEO, and 100 µg/mL ampicillin as required.

4.2. Screening for Spontaneous BTZ-Resistant Msm Strains

Msm cultures at an OD₆₀₀ of 1.0 were plated (500 µL) on 7H10 medium containing 100, 200, or 300 µg/mL BTZ, with two replicates per concentration. Serial tenfold dilutions were then plated on BTZ-free 7H10 agar to calculate colony-forming units (CFU). The mutation frequency was determined by counting CFU and the number of resistant colonies. Resistant strains were analyzed for mutations in *prcBA* and *clpP1P2* by Sanger sequencing using primers listed in Table S5. Genomic DNA from the parent strain and seven BTZ-resistant strains (which did not harbor mutations in *prcA*, *prcB*, *clpP1*, or *clpP2*) was sequenced by Shanghai Jingnuo Biotechnology Co. Mutations in the resistant strains were identified by comparing their genomic profiles to that of the parent strain (Table S4).

4.3. Construction of Knockout Strains

Gene knockout was performed using CRISPR-Cpf1-assisted recombineering technology [30]. First, the pJV53-Cpf1 plasmid was electroporated into Msm to obtain Msm::pJV53-cpf1 and then induced competent cells were prepared. The crRNA sequences were designed using the website (<https://chopchop.cbu.uib.no/>), synthesized by Sangon Biotech Co., Ltd. (Table S6), and ligated into the linearized pCR-Zeo vector after annealing. Approximately 800 bp upstream and downstream fragments (including 15-36 bp of the target gene to be deleted) were amplified by PCR and assembled into the pBlueSK vector, followed by PCR amplification of the resulting ~1600 bp double-stranded template. The pCR-Zeo plasmid containing the crRNA sequence and the double-stranded template were then electroporated into the Msm::pJV53-cpf1 inducible competent cells. The cells were plated on 7H11 agar containing 50 µg/mL KAN, 30 µg/mL ZEO, and 200 ng/mL aTc and incubated at 30°C. Positive colonies were identified by PCR and Sanger sequencing.

4.4. Construction of Overexpression and Complementation Strains

The target genes were amplified from Msm and cloned into the linearized pRH2502 plasmid under the control of either the exogenous *hsp60* promoter or the native promoter of the respective genes. Primers used in this study are listed in Table S5. The recombinant plasmids were electroporated into either wild-type Msm (for overexpression strains) or the corresponding knockout strains (for complementation strains). Transformants were plated on 7H10 agar containing 50 µg/mL KAN, and positive colonies were confirmed by PCR.

4.5. Construction of Gene Editing Strains

CRISPR-Cpf1-assisted recombineering [30] was used for gene editing. CrRNAs targeting the mutation sites were designed using the CHOPCHOP web tool (Table S6) and subsequently into the linearized pCR-Zeo vector. The 500 bp flanking regions (~1000 bp total) of the mutation site were amplified from the resistant strains and used as repair templates. The crRNA-containing pCR-Zeo plasmid and repair templates were co-electroporated into Msm::pJV53-Cpf1 inducible competent cells. Transformants were plated on 7H11 agar containing 50 µg/mL KAN, 30 µg/mL ZEO, and 200 ng/mL aTc and incubated at 30°C. Positive colonies were confirmed by PCR and Sanger sequencing.

4.6. DST

In this study, all drugs were prepared in solutions with DMSO or water at a final concentration of 10 mg/mL, except for CFZ, which was prepared at a final concentration of 5 mg/mL. Two methods were used to test the drug susceptibility of the strains. Knockout, overexpression, and complementation strains were grown in 7H9 medium at 37°C to the logarithmic phase for both methods.

For the microplate broth dilution assay, cultures were adjusted to an OD₆₀₀ of 0.5 and diluted 1:1000. The MICs were determined using the broth microdilution method in 96-well plates. Cultures were incubated at 37°C for 72 hours, and the MIC was defined as the lowest concentration of antibiotic that visibly inhibited bacterial growth [17].

For the solid plate assay, cultures were adjusted to an OD₆₀₀ of 0.9. Bacterial suspensions were serially diluted in 10-fold increments to obtain six different dilutions, ranging from the undiluted suspension to a 10⁻⁵ dilution. A 1 µL aliquot of each dilution was spotted onto solid media containing different concentrations of drugs, with 7H10 solid media without antibiotics serving as the control. The plates were incubated at 37°C for three days, and colony growth was assessed visually and photographed.

4.7. Cell Wall Permeability Assay

EtBr accumulation assay was performed as described previously [36]. The EtBr working solution (4 µg/mL) was prepared in PBS containing 0.08% glucose and 0.05% Tween 80 was prepared. Cultures were grown to an OD₆₀₀ of 0.6–0.8, centrifuged, and resuspended in PBS with 0.05% Tween 80 to an OD₆₀₀ of 0.5. For each strain, 100 µL of the cell suspension was added to a white 96-well plate (3 experimental wells, 3 no-reagent controls, and 3 no-cell controls). Fluorescence was measured

using a multimode microplate reader (excitation: 530 nm, emission: 590 nm) at 1-minute intervals over 60 minutes. Background fluorescence was subtracted, and data were analyzed using GraphPad Prism 8.0.2. Results are presented as the mean $\pm$ SD, with $n=3$ for each group. Statistical significance was assessed using an independent t -test, with $P < 0.05$ considered significant.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: title; Table S1: title; Video S1: title.

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