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Mutations in the *embC-embA* Intergenic Region Contribute to *Mycobacterium tuberculosis* Resistance to Ethambutol

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The rapid increase in *Mycobacterium tuberculosis* resistance to ethambutol (EMB) threatens the diagnosis and treatment of tuberculosis (TB). We investigated the role of mutations in the *embC-embA* intergenic region (IGR) in EMB-resistant clinical strains from east China. A total of 767 *M. tuberculosis* clinical strains were collected and analyzed for their drug susceptibility to EMB using the MGIT 960 system and MIC assay, and the *embC-embA* IGRs of these strains were sequenced. The transcriptional activity of the *embC-embA* IGR mutations was examined by reporter gene assays in recombinant *Mycobacterium smegmatis* strains, and the effect of IGR mutations on its binding to EmbR, a transcription regulator of *embAB*, was analyzed by gel mobility shift assays. Correlation coefficient analysis showed that the *embC-embA* IGR mutation is associated with EMB resistance. The clinical strains carrying IGR mutations had a much higher level of *embA* and *embB* mRNA as well as higher MICs to EMB. IGR mutations had higher transcriptional activity when transformed into *M. smegmatis* strains. Mutated IGRs bound to EmbR with much higher affinity than wild-type fragments. The sensitivity of molecular drug susceptibility testing (DST) with IGR mutations as an additional marker increased from 65.5% to 73.5%. Mutations of the *embC-embA* IGR enhance the binding of EmbR to the promoter region of *embAB* and increase the expression of *embAB*, thus contributing to EMB resistance. Therefore, identification of IGR mutations as markers of EMB resistance could increase the sensitivity of molecular DST.

Tuberculosis (TB) remains a major global health problem. It affects millions of people each year and is the second leading cause of death from an infectious disease worldwide. In 2012, an estimated 8.6 million people developed TB, and 1.3 million died from the disease. The emergence of drug-resistant strains of *Mycobacterium tuberculosis*, especially those that are multidrug resistant (MDR) and extensively drug resistant (XDR), has posed a serious threat to global TB control programs (1). An estimated 3.6% of new patients and 20.2% of previously treated patients have MDR-TB, and there were 450,000 new cases of MDR-TB worldwide in 2012 (2). Given the alarming rise of drug-resistant TB, the identification of drug resistance genes is critical for the detection and treatment of TB. Much progress has been made to identify gene mutations in specific loci of the *M. tuberculosis* genome as the molecular basis for TB drug resistance and as drug targets for the development of anti-TB drugs. Although an association of mutations in these resistance genes with drug resistance has been observed, the exact role these genes play in the development of drug resistance is not fully understood. Furthermore, a significant number of anti-TB drug-resistant strains do not carry these mutations, suggesting that unknown gene mutations or variations are involved in the development of anti-TB drug resistance.

Ethambutol (EMB) is an essential first-line anti-TB drug that inhibits the biosynthesis of cell wall arabinogalactan (3). The resistance rate to EMB has gradually increased in some regions and approaches 50% in re-treated TB patients (4–6). In China, the resistance rate to EMB increased from 6.52% in 2007 to 17.18% in 2010 (7). Resistance to EMB is caused by mutation of the *embCAB* operon (*embC*, *embA*, and *embB*) that encodes membrane-associated arabinosyltransferases involved in the synthesis of cell wall arabinogalactan. Approximately 50% to 70% of EMB-resistant clinical *M. tuberculosis* isolates carry a mutation in a relatively short region in *embB*, primarily at codons 306 (*embB306*), 406 (*embB406*), and 497 (*embB497*), which therefore represent promising diagnostic markers for the rapid detection of EMB resistance (8–14). However, allelic-exchange studies indicate that *embB306*, *embB406*, and *embB497* mutations only modestly increase resistance to EMB in *M. tuberculosis* (15, 16). Some mutations of the *embC-embA* intergenic region (IGR) have been identified in EMB-resistant clinical strains, but the molecular basis of these mutations in the regulation of EMB resistance is not well characterized (10, 17). In this study, we analyzed the mutations in *embC-embA* IGR in 767 clinical isolates of *M. tuberculosis* strains and verified the effects of mutations in *embC-embA* IGR on EMB resistance in *M. tuberculosis*.

MATERIALS AND METHODS

Strains. A total of 767 *M. tuberculosis* clinical strains were randomly collected from patients with pulmonary tuberculosis. All of the pulmonary tuberculosis patients were from east China. The clinical strains were identified as *M. tuberculosis* using PCR for the IS*6110* sequence (18).

DST. All isolates were initially classified as EMB resistant or susceptible in routine diagnostic laboratories by the Bactec MGIT 960 method (5 μg/ml) (19). All strains were cultured in a mycobacterial growth indicator tube (MGIT) with the Bactec MGIT 960 growth supplement (Becton Dickinson Diagnostic Systems, MD). We used the MGIT 960 instrument and the EpiCenter software package (Becton Dickinson Diagnostic Systems, MD). The standard protocol for susceptibility testing in MGIT 960 was strictly followed, as recommended for primary drugs. The Bactec MGIT 960 drug susceptibility testing (DST) supplement (0.8 ml) (oleic acid-albumin-dextrose-catalase), 100 μl of the drug stock solution, and

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0.5 ml of the suspension containing *M. tuberculosis* were added to an MGIT tube. The growth control did not contain the drug stock solution. DST sets were entered into the Bactec MGIT 960 instrument and continuously monitored until a susceptible or resistant result was obtained. The DST set results were reported by the instrument (determined by the software algorithms, after the growth control became positive).

MIC testing. To determine EMB MICs, susceptibility testing was performed with the microplate alamarBlue assay (MABA) using Middlebrook 7H9 medium (Becton Dickinson Diagnostic Systems, Sparks, MD), including a 10% albumin-dextrose-catalase (ADC) supplement and EMB at concentrations of 0, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/ml, with reference to the method of Franzblau et al. (20).

PCR and sequencing. One milliliter of *M. tuberculosis* suspension collected from an MGIT 960 control tube was transferred to a 1.5-ml tube and centrifuged at 10,000 × *g* for 5 min. The supernatant was discarded, and the sediment was resuspended in 50 μl DNA extraction solution (0.04% NaOH, 0.1% SDS, 15% Chelex-100 chelating resin) and mixed by vortexing. Subsequently, the tube was incubated at 100°C for 15 min and centrifuged at 13,000 × *g* for 10 min after it had cooled. Finally, the supernatant was transferred to a fresh1.5-ml tube and preserved at −20°C until used as a PCR template.

The *embC-embA* IGR was amplified with primer 1 (5′-GGTTGACGC CTTACTACCC-3′) and primer 2 (5′-CCACGACGACCGTGTCC-3′). The *embB* mutation hot region (including codons 306, 406, and 497) was amplified with primer 3 (5′-CTGAAACTGCTGGCGATCAT-3′) and primer 4 (5′-ATAGCGCGGTGATCAAAAAG-3′). These primers were designed by Primer-BLAST software with reference to *embABC* (GenBank accession no. NC_000962.2) gene sequences of *M. tuberculosis* H37Rv. The sizes of the amplified fragments were 535 bp for *embC-embA* IGR and 997 bp for *embB*. The PCR products were purified and sequenced at the Beijing Genomics Institute (BGI [Shenzhen, China]). The DNA sequences were analyzed with MegAlign 5.01 software (demonstration system; DNAStar, Inc., Madison, WI).

Quantitative real-time PCR of *embAB* mRNA. All of the strains with *embC-embA* IGR mutations and 15 randomly selected EMB-susceptible strains without *embC-embA* IGR and *embB* mutations were cultured in a mycobacterial growth indicator tube (MGIT) with Bactec MGIT 960 growth supplement. After 1 week, all strains were in log phase. The strains were collected, and total RNA was extracted immediately. RNA was extracted as described previously (21). The genomic DNA was removed using the PrimeScript reverse transcription (RT) reagent kit with gDNA Eraser (TaKaRa Biotechnology Co., Ltd. Inc., Dalian, China). RT was carried out using random primers. The reaction was carried out with SBGR reverse transcription reagents (Tiangen Biotech Co., Ltd., Beijing, China). The primers for *embA* (5′-CTACGGGGAGAACAACCTGG-3′ and 5′-CCACTGCAGTTTCAGGGACT-3′) and *embB* (5′-ATGCTGCG GATCTTGGTGCG-3′ and 5′-CCGATTTTGGCGCGAACCCT-3′) and the primers for 16S rRNA (5′-GTCAAGTCATCATGCCCCTT-3′ and 5′-CACCTTCGACAGCTCCCTCC-3′) were used in real-time PCR assays. *embAB* expression was normalized against the 16S rRNA housekeeping gene.

Analysis of promoter activity. The *embC-embA* IGR of *M. tuberculosis* was amplified using primer 5 (5′-CGCTTCTAGAAGCGGTTGACGCCT TACTAC-3′) and primer 6 (5′-TCTGGATCCAGATCGCTCATTACCG TCGT-3′) with XbaI and HindIII restriction endonuclease sites and was cloned upstream of the promoterless *lacZ* gene of vector pMC210 (a kind gift from Xiaoyong Fan). The recombinant pMC210-IGR plasmid was isolated and the sequence verified. Plasmids pMC210-IGR with the wild-type and different mutant types of *embC-embA* IGR were electroporated into *Mycobacterium smegmatis*. Kanamycin-resistant transformants were isolated. Three independent transformants for each were selected for promoter activity determinations.

β-Galactosidase activity was assayed *in vitro* in mycobacteria (22). Briefly, the recombinant mycobacterial strains were grown in complete Middlebrook 7H9 broth to the log phase, and the optical density at 600 nm (OD_{600}) was measured. One hundred microliters of cells was added to 900 μl of Z buffer (6 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) and permeabilized with 50 μl of 0.1% SDS and 100 μl of chloroform. The mixtures were vortexed and then incubated for 15 min at room temperature. Two hundred microliters of substrate *o*-nitrophenyl-β-D-galactosidase (ONPG [4 mg/ml in 100 mM KH_2PO_4, pH 7.0]) was added, and the time taken for yellow color development was noted. The reaction was halted by addition of 0.2 ml of 2.5 M Na_2CO_3. The solutions were centrifuged to remove cell debris, and the optical density at 420 nm was measured. The β-galactosidase activity in modified Miller units was calculated with the following formula: $(OD_{420} \times 1{,}000)/(t \times v \times OD_{600})$, in which t is the incubation time in minutes, v is the volume of culture in ml, and OD_{420} or OD_{600} is the optical density at 420 or 600 nm, respectively.

Gel mobility shift assay (EMSA). The electrophoretic mobility shift assay (EMSA) was performed as follows. The expression and purification of EmbR and the phosphorylation of EmbR by PknH followed the method of Molle et al. (23). For the protein-DNA binding assay, a DNA fragment was purified as a PCR product. The primers were 5′-GAAGTGCCGTGG CCACCGAC-3′ and 5′-CGTGCGATCCGGTGAGATCG-3′. The PCR products representing different promoter regions were incubated with constant amounts of phosphorylated EmbR at 4°C for 30 min in buffer containing 10 mM Tris-HCl (pH 7.0), 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol in a total volume of 10 μl. After incubation, complexes and free DNA were resolved by 5% nondenaturing polyacrylamide gels with a running buffer containing 40 mM Tris-HCl (pH 7.8), 20 mM sodium acetate, and 1 mM EDTA. The gels were then stained with SYBR green, and the band intensity was analyzed by Gel-Pro analysis software (Media Cybernetics, Inc.).

Data analysis. Data analysis was carried out using GraphPad Prism 5 (GraphPad Software, Inc., CA). The mRNA transcriptional level and the MIC of EMB were compared with the Mann-Whitney *U* test. Comparison of sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) was performed using a chi-square test.

RESULTS

***embB* mutations in EMB-resistant strains.** We collected 767 epidemiologically unrelated *M. tuberculosis* clinical strains from TB patients in east China. Of the 767 clinical strains, 275 strains were found to be EMB resistant. In the 275 EMB-resistant strains, only 180 (65.5%) carried known EMB resistance-associated *embB* mutations at codons 306, 406, and 497. Sixteen (5.8%) of the EMB-resistant strains carried other *embB* site nonsynonymous mutations (Table 1).

***embC-embA* IGR mutations in *M. tuberculosis* clinical strains.** Among the 767 clinical strains, 57 strains (7.43%) were found to carry IGR mutations, located at positions −8, −11, −12, −16, −27, and −41 upstream of *embA* (Fig. 1 and Table 2). Of the 57 clinical strains carrying IGR mutations, 52 (91.22%) were found to be EMB resistant, as determined by the Bactec MGIT 960 method (5 mg/ml) (19). In 275 EMB-resistant strains, 149 strains carried only the *embB* mutation (e.g., site 306, 406, or 497), 31 strains carried both *embC-embA* IGR and *embB* mutations, and 22 strains carried only *embC-embA* IGR mutations. Correlation coefficient analysis of all 767 clinical strains showed that the *embC-embA* IGR mutation is associated with EMB resistance ($r = 0.3375$, $P < 0.0001$), suggesting that mutations of IGR may play a role in EMB resistance.

***embAB* mRNA transcription in clinical isolates.** To examine whether the IGR mutation is associated with the expression of *embAB*, the mRNA levels of *embA* and *embB* in all EMB-resistant clinical strains with *embC-embA* IGR mutations and 15 randomly selected EMB-susceptible strains without *embC-embA* IGR and

TABLE 1 Mutation of *embA* and *embB* in *M. tuberculosis* clinical strains

embB mutation codon substitution		No. of strains with EMB DST result[a]:		*embA* mutation nucleotide substitution or deletion		No. of strains with EMB DST result[a]:	
Site(s)	Pattern	S	R	Site	Pattern	S	R
306	M-I	23	34	−12	C-T		2
				−16	C-A		1
				−16	C-T		1
				−16	C-G		7
	M-L	6	10	−11	C-A		2
				−12	C-T		1
				−16	C-A		2
				−16	C-T		1
	M-V	13	90	−11	C-A		1
				−16	C-T		2
				−16	C-G		1
406	G-S	7	2	−11	C-T		1
	G-R		1				
	G-D	11	3	−12	C-T		1
	G-A	4	15	−16	C-T		1
				−16	C-G		3
497	G-C		1				
	Q-P	1					
	Q-R	3	7	−16	C-T	1	1
				−41	A del		3
	Q-K	1	5				
306 and 406	M-I and G-D		3				
	M-I and G-A		2				
	M-V and G-D		2				
306 and 497	M-I and Q-F		1				
	M-I and Q-R		1				
296 and 306	N-I and M-I		1				
304 and 306	L-V and M-I		1				
360 and 406	V-A and G-A		1				
246	G-R	1	4				
288	L-V		1				
296	N-S	4					
319	Q-R		1	−12	C-T		1
328	D-G		1				
328	D-F	1					
328	D-Y	1	1				
330	F-L	1	4				
334	Y-H	1	0				
354	D-A	3	3	−12	C-T	1	
380	S-N	1					
397	P-R		1				
397	P-S	1		−16	C-T	1	0
400	N-S	1					
409	A-P	1					
445	E-R	1					
446	L-S	1					
466	L-W	1					
Detection region	wt[b]			−8	C-T		1
				−11	C-A		3
				−12	C-T	1	9
				−16	C-T		6
				−16	C-G		1
				−27	T del		1

[a] R, resistance; S, susceptibility.
[b] wt, *embB* in the detection region was wild type.

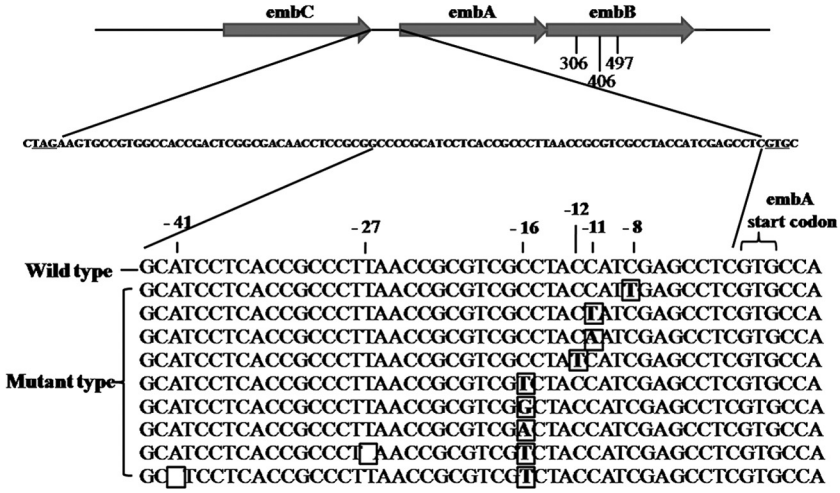

FIG 1 Alignment of the promoter sequence of *embA* in *M. tuberculosis* clinical isolates. −8, −11, −12, −16, −27, and −41 mutations of the *embA* promoter were identified in *M. tuberculosis* clinical isolates resistant to EMB. Mutations are marked in boldface in a box; a blank box indicates nucleotide deletion.

embB mutations were analyzed by RT-PCR (Fig. 2). The transcription levels of *embA* and *embB* mRNA in EMB-resistant strains with only *embC-embA* IGR mutations were higher than those in EMB-susceptible stains without *embC-embA* IGR or *embB* mutations ($P = 0.0011$, $P = 0.0003$) by the Mann-Whitney *U* test. In EMB-resistant strains, the levels of *embA* and *embB* mRNA with only *embC-embA* IGR mutations were higher than those in EMB-resistant strains with only the *embB* mutation ($P < 0.0001$, $P = 0.0006$). The levels of *embA* and *embB* mRNA with both *embC-embA* IGR mutations and *embB* mutations were higher than those with only *embB* mutations ($P = 0.0002$, $P = 0.0075$). These results suggest that *embC-embA* IGR mutations may increase the transcription of downstream *embA* and *embB*.

Effect of *embC-embA* IGR mutations on resistance to EMB. We compared the MICs to EMB of those *M. tuberculosis* clinical strains with simultaneous *embC-embA* IGR and *embB* mutations by the microplate alamarBlue assay (20). The MICs of EMB of those strains with both *embC-embA* IGR mutations and *embB* mutations were much higher than those of strains with only an *embB* mutation ($P = 0.0004$) (Fig. 3). However, only the MICs of clinical strains with an *embC-embA* IGR mutation were similar to those of strains with only an *embB* mutation ($P = 0.5313$).

TABLE 2 Mutation of the *embC-embA* IGR in *M. tuberculosis* clinical strains

Mutation sites	Mutation pattern	No. of strains	MGIT EMB DST result[a]	Median MIC, μg/ml (interquartile range [%])
−8	C-T	1	R	4 (4–4)
−11	C-A	6	R	8 (2–8)
	C-T	1	R	4 (4–4)
−12	C-T	14	R	4 (4–8)
	C-T	2	S	4 (4–4)
−16	C-G	12	R	8 (2–16)
	C-T	12	R	4 (4–8)
	C-T	2	S	4 (4–4)
	C-A	3	R	8 (8–8)
−27	T del	1	R	2 (2–2)
−41	A del	3	R	8 (8–8)

[a] R, resistance; S, susceptibility.

The transcriptional activity of promoters of *embAB* in *M. smegmatis*. To further analyze whether IGR mutations affect transcriptional activity, the IGR mutation region was cloned into the vector pMC210 upstream of the promoterless *lacZ* gene of vector pMC210 (19). The recombinant *M. smegmatis* strains transformed with *embC-embA* IGR mutation construct vectors (mutations of −8C to T [−8C-T], −11C-A, −11C-T, −12C-T, −16C-A, and −16C-T and the −27T deletion) had higher levels of β-galactosidase activity than the recombinant *M. smegmatis* with wild-type *embC-embA* IGR construct vectors (Fig. 4). Of all mutant types of *embC-embA* IGR, the −16C-A mutant had the highest level of β-galactosidase activity, which was almost 4-fold higher than that of the wild type.

Binding activity of EmbR to an *embC-embA* IGR fragment. EmbR is a multidomain protein possessing three major domains: a DNA binding winged helix-turn-helix (W-HTH) domain, a bacterial transcription activation domain, and a forkhead-associated (FHA) domain (23). Phosphorylation of EmbR by mycobacterial serine/threonine kinase PknH enhances its DNA binding activity toward promoter regions of *embCAB*, thus regulating transcription of *embCAB* genes in *M. tuberculosis* (24). Interestingly, a mutation in the EmbR FHA domain was shown to be associated with EMB resistance (10). EMSA revealed that pEmbR binds to wild-type *embC-embA* IGR fragments with much lower affinity than other mutated *embC-embA* IGR fragments. This result suggested that mutant *embC-embA* IGR increased the binding activity of EmbR to *embC-embA* IGR (Fig. 5).

The value of the *embC-embA* mutation in the diagnosis of molecular DST to EMB. With reference to the MGIT 960 method, if *embB* mutations (including only known sites related to EMB resistance: codons 306, 406, and 497) were used as a judgment standard of molecular DST to EMB, the sensitivity, specificity, and accuracy in this study were 65.5% (180/275), 86.0% (423/492), and 78.6% (603/767). The positive predictive value (PPV) and the negative predictive value (NPV) were 72.3% (180/249) and 81.7%

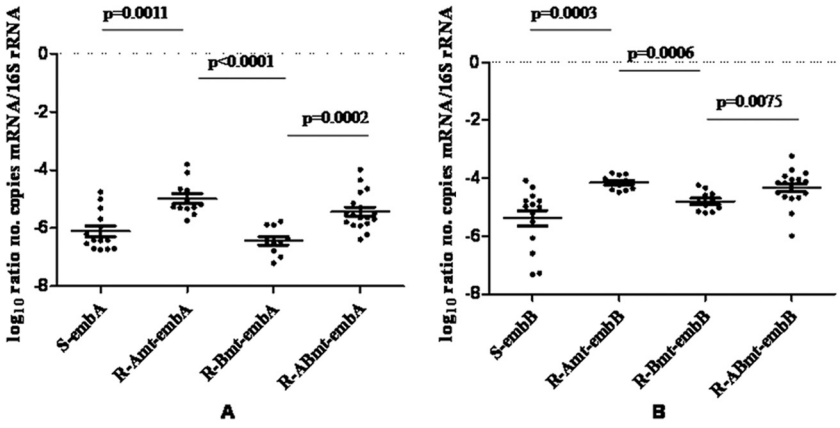

FIG 2 *embC-embA* IGR mutations lead to increased *embA* and *embB* mRNA expression in clinical strains. R, resistant to EMB; S, susceptible to EMB; Amt, only *embC-embA* IGR mutation; Bmt, only *embB* mutation; Abmt, both *embC-embA* IGR and *embB* mutations.

(423/518), respectively. If *embC-embA* IGR mutations were used as a reference standard of molecular drug susceptibility testing for EMB, the sensitivity, specificity, accuracy, PPV, and NPV were 19.3% (53/275), 99.2% (488/492), 70.5% (541/767), 93.0% (53/57), and 68.7% (488/710), respectively. If either of the *embB* or *embC-embA* IGR mutations was used as a reference standard of molecular DST for EMB, the sensitivity, specificity, accuracy, PPV, and NPV were 73.5% (202/275), 85.4% (420/492), 81.1% (622/767), 73.7% (202/274), and 85.2% (420/493), respectively. The PPV of the *embC-embA* IGR mutations was significantly higher than that of the *embB* mutations ($\chi^2 = 9.895$, $P = 0.001$).

DISCUSSION

With the rapid increase in EMB resistance in *M. tuberculosis* clinical strains, a rapid and accurate method is urgently needed for the diagnosis of EMB resistance. Molecular DST has shown good performance in the diagnosis of rifampin resistance, but the sensitivity and specificity of *embB*-based molecular DST of EMB resistance are lower, suggesting that other gene mutations are involved in EMB resistance. Previous studies by several groups have detected mutations in *embC-embA* IGR in strains from the United States, the former Soviet Union, Kuwait, Germany, and Uzbekistan but found no epidemiological correlation of these mutations with EMB resistance, possibly due to there being fewer clinical strains showing mutations in *embC-embA* IGR (10, 25, 26). In this study, we found *embC-embA* IGR mutations in clinical EMB-resistant strains from east China, suggesting a wide distribution of *embC-embA* IGR mutations across the world. Moreover, our analysis of a large number of clinical strains showed that mutations of *embC-embA* IGR were highly related to resistance to EMB in *M. tuberculosis* clinical strains.

Overexpression of *embAB* is associated with high-level EMB-resistant arabinosyltransferase activity (27). In our study, a higher level of *embA* and *embB* mRNA expression was found in strains with *embC-embA* IGR mutations than in strains without *embC-embA* IGR mutations, suggesting that the mutations in the *embC-embA* IGR may increase the transcriptional level of *embA* and *embB*. Indeed, the promoter activities of most of the mutant *embC-embA* IGRs were higher than those in the wild type, as as-

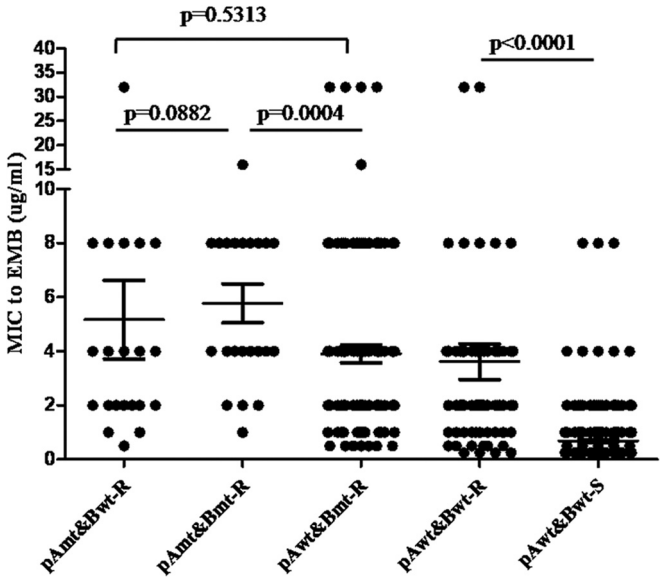

FIG 3 *embC-embA* IGR mutations confer a higher resistance to EMB in clinical strains. pAmt, mutation in *embC-embA* IGR; Bmt, mutations in *embB* site 306, 406, or 497; pAwt, wild type in *embC-embA* IGR; Bwt, wild type in *embB* site 306, 406, or 497; R, EMB-resistant strains; S, EMB-susceptible strains.

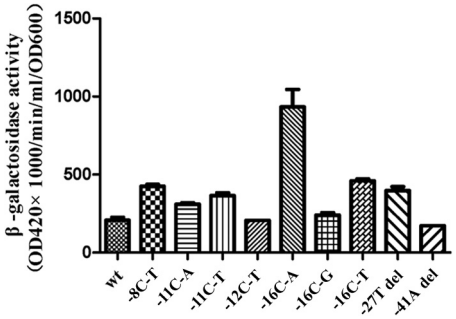

FIG 4 *embC-embA* IGR mutations as promoters of *embA* and *embB* increase the transcriptional activity. β-Galactosidase activities in *M. smegmatis* recombinants with mutant *embC-embA* IGR mutation construct vectors (−8C-T, −11C-A, −11C-T, −16C-A, −16C-T, and −27T deletion) were higher than those in the recombinant with the wild-type construct vector ($P = 0.0049$, 0.0049, 0.0024, 0.0049, 0.0049, and 0.0049, respectively).

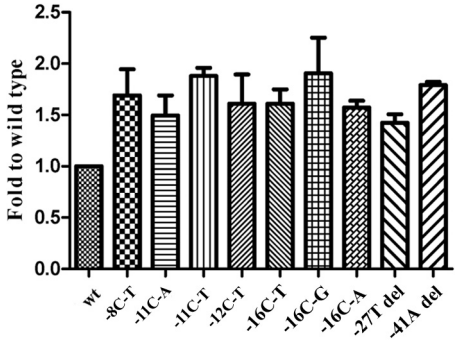

FIG 5 Binding activity of EmbR to *embC-embA* IGR. EmbR binds to wild-type *embC-embA* IGR fragments with much lower affinity than the mutant (−8C-T, −11C-A, −11C-T, −12C-T, −16C-A, −16C-T, −16C-G, −27T deletion and 41A deletion) *embC-embA* IGR fragments ($P = 0.0107$, 0.0172, 0.0020, 0.0150, 0.0059, 0.0013, 0.0342, 0.0090, and 0.0092, respectively).

sayed by a β-galactosidase activity reporter system in *M. smegmatis*. Of all 10 mutant genotypes of *embC-embA* IGR, the promoter activity of the −16C-A mutation is the highest. These results suggest that mutations of *embC-embA* IGR may increase the expression of *embAB*, thus contributing to EMB resistance.

It was reported that mutations in *embB* codons 306, 406, and 497 only cause EMB resistance at a low level of EMB (15, 16). Safi et al. reported that synonymous mutations in Rv3792 increased the expression of *embC* to increase the resistance level of EMB (28). We found that the MIC values for EMB in clinical strains with mutations in both *embC-embA* IGR and *embB* were significantly higher than those in strains carrying only the *embB* mutation. Moreover, the levels of *embA* and *embB* mRNA with both *embC-embA* IGR mutations and *embB* mutations were higher than those with only the *embB* mutation. Thus, mutations in *embC-embA* IGR may increase EMB resistance through enhancement of the transcription of *embA* and *embB*, thus providing a novel mechanism of regulating the resistance level to EMB in *M. tuberculosis* clinical strains. In this study, the breakpoint of the MABA DST method was 1 μg/ml. When the MIC was more than 1 μg/ml, the strain was considered to be EMB resistant. Therefore, the MICs of some EMB-resistant strains by MGIT (Table 2) were less than 5 μg/ml, such as 2 μg/ml and 4 μg/ml. The MICs of four EMB-susceptible strains with *embC-embA* IGR mutations (−12C-T and −16C-T) judged by MGIT were more than 1 μg/ml. Thus, according to the results of MABA DST, higher transcriptional activity of these two mutant sites and higher binding affinity of *embR*, two strains with −12C-T and two strains with −16C-T should better be judged resistant strains.

The binding of EmbR to the *embABC* promoter region regulates transcription of the *embCAB* operon and increases the MIC to EMB (29). Our results showed that the binding activity of EmbR to mutant *embC-embA* IGR fragments was higher than that to the wild-type *embC-embA* IGR, suggesting that mutations in *embC-embA* IGR may facilitate the binding of EmbR to the promoter region of *embAB* to enhance *embA* and *embB* mRNA transcription, thus contributing to *M. tuberculosis* resistance to EMB. Whether *embC-embA* IGR mutations also increases the binding of additional transcriptional regulators to the *embABC* promoter region requires further investigation. Our data revealed that mutation of *embC-embA* IGRs increases its binding with phosphorylated EmbR (Fig. 5). We have also found that incubation of EmbR with PknH enhances its binding with either wild-type or *embC-embA* IGRs (data not shown). Thus, it appears that the binding of EmbR with *embC-embA* IGRs depends on two aspects: one is the phosphorylation state of EmbR, which could be induced by specific stimuli or autoactivation, and another is the specific sequence of *embC-embA* IGRs.

Molecular drug susceptibility testing based on *embB* mutations as a rapid method was used to test the drug susceptibility of *M. tuberculosis* to EMB. However, due to the low mutation rate of *embB* in EMB-resistant *M. tuberculosis* strains, the sensitivity of molecular drug susceptibility testing based on *embB* was too low to satisfy the needs of clinical diagnosis. According to our data, combining *embC-embA* IGR with *embB* as a diagnostic marker for drug susceptibility testing can increase sensitivity and NPV.

In summary, our data demonstrate that mutations in the *embC-embA* IGR contribute to *M. tuberculosis* resistance to EMB. These *embC-embA* IGR mutations could be used as a marker of molecular DST to enhance the sensitivity of molecular tests for drug resistance and also as an indicator of the drug resistance level in some clinical strains with *embB* mutations.

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