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rmlB and *rmlC* genes are essential for growth of mycobacteria

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Abstract

The rhamnose-GlcNAc disaccharide linker is fundamental to the structural integrity of mycobacterial cell wall. The donor dTDP-rhamnose is synthesized by four enzymes (RmlA, B, C, and D) beginning with dTTP and glucose-1-phosphate. We generated *M. smegmatis rmlB* gene knockout mutant (transcription of downstream *rmlC* gene was blocked because of a polar effect) by homologous recombination. When the *Mycobacterium tuberculosis* (Tb) *rmlB* rescue plasmid carrying a temperature-sensitive replication origin and Tb *rmlC* bearing plasmid with a normal replication origin were present in the mc^{2}155 *rmlB* knockout mutant, the mutant was unable to grow at non-permissive temperature (42 °C) where the Tb *rmlB* rescue plasmid is lost. While the Tb *rmlC* rescue plasmid carrying a temperature-sensitive replication origin and Tb *rmlB* bearing plasmid with a normal replication origin were present in the mc^{2}155 *rmlB* knock out mutant, this mutant was also unable to grow at the non-permissive temperature where the Tb *rmlC* rescue plasmid is lost. These results demonstrate that *rmlB* and *rmlC* genes are essential for mycobacterial growth, therefore, RmlB and RmlC are essential targets to develop new anti-tuberculosis drugs.

Keywords: *M. tuberculosis*; *M. smegmatis*; Cell wall; dTDP-D-glucose-4,6-dehydratase; RmlB; dTDP-4-keto-6-deoxyglucose-3,5-epimerase; RmlC; Gene knockout

The major components of the mycobacterial cell wall include mycolic acid, arabinogalactan, and peptidoglycan [1,2] (Fig. 1B). The mycolic acid lipid layer is attached to the peptidoglycan layer by means of the polysaccharide arabinogalactan (AG). AG contains three regions: a D-arabinofuran region, a D-galactofuran region, and the disaccharide linker, α-L-rhamnosyl-(1 → 3)-α-D-*N*-acetyl-glucosaminosyl-1-phosphate. The phosphate group is linked to a muramic acid residue of the peptidoglycan, the galactofuran is attached to the L-rhamnosyl residue of the linker, and the mycolated arabinofuran is attached to the galactan. Therefore, the disaccharide linker is fundamental to the structural integrity of the cell wall and thus required for mycobacterial viability.

The L-rhamnosyl residue in the linker region is provided with a sugar donor, dTDP-rhamnose. The biosynthetic pathway of dTDP-rhamnose consists of four-step reactions from α-D-glucose-1-phosphate and TTP to dTDP-rhamnose through three intermediates [3–6] (Fig. 1A). Four reactions are catalyzed by four enzyme activities of α-D-glucose-1-phosphate thymidylyltransferase (RmlA), dTDP-D-glucose-4,6-dehydratase (RmlB), dTDP-4-keto-6-deoxyglucose-3, 5-epimerase (RmlC), and dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase (RmlD), respectively. The *rmlA-D* genes for the RmlA-D enzymes are located together as rml gene cluster in many Gram-negative and Gram-positive bacteria, e.g., *Escherichia coli* [4], *Salmonella enterica* [7], *Vibrio cholerae* [8], *Streptococcus mutans* [9], etc. However, *Mycobacterium tuberculosis rmlA-D* genes (Fig. 1C) are not located in a locus in the genome [10]. The *rmlA* (Rv0334) gene is isolated from any other rhamnosyl formation enzymes, the *rmlB* (Rv3464) and *rmlC* (Rv3465) genes are together in an operon, and *rmlD*

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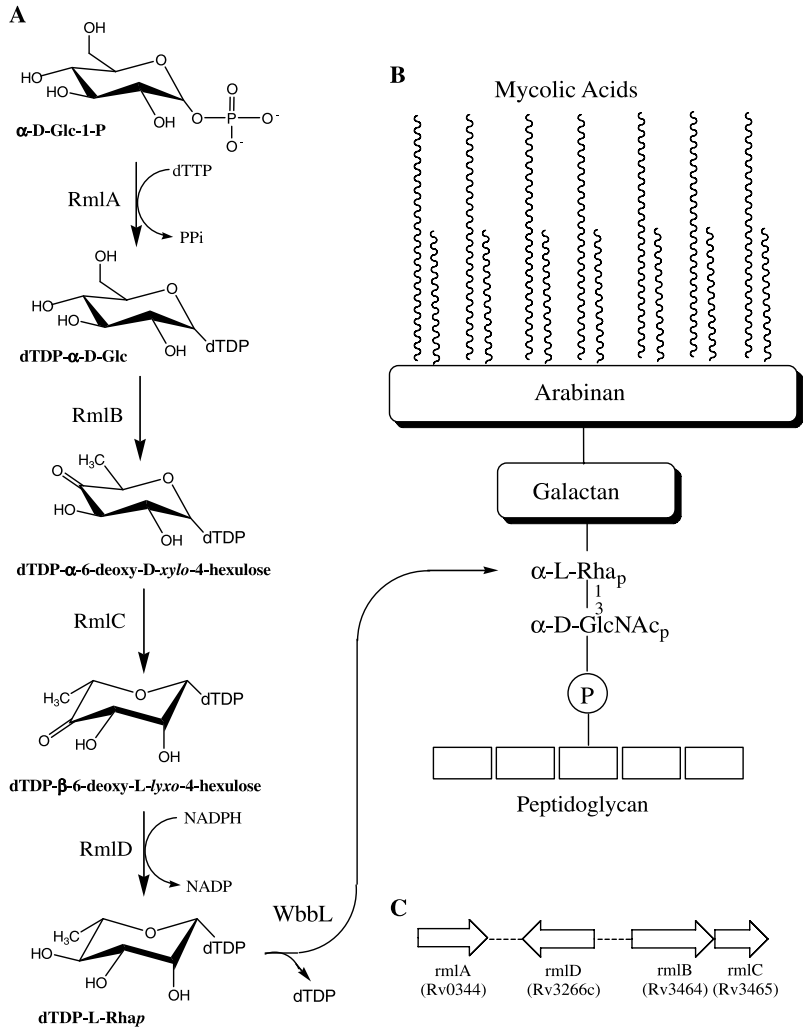

Fig. 1. (A) The biosynthesis pathway of dTDP-Rha. (B) The structure of AG. (C) The gene cluster containing *rmlA*, *rmlB*, *rmlC*, and *rmlD* genes in *M. tuberculosis* H37Rv genome.

(Rv3266c) gene is found in an operon with *wbbL* (Rv3265c) and *manB* (Rv3264c) [10]. The rhamnosyl transferase encoded by *wbbL* gene transfers the rhamnosyl residue of dTDP-rhamnose into D-*N*-acetyl-glucosaminosyl-1-phosphate to form a disaccharide linker.

Mycobacterium tuberculosis RmlA-D enzymes utilized in the biosynthesis of dTDP-rhamnose for disaccharide linker formation have been expressed in *E. coli* [11–13] and RmlB-D assay has been established to screen inhibitors for the development of new tuberculosis therapeutics [11]. Therefore, it is important to directly demonstrate that any enzyme involved in dTDP-rhamnose formation is a valid drag target. The complementation of *M. smegmatis rmlD* gene knockout strains by *M. tuberculosis rmlD* gene indicates that RmlD is essential for mycobacterial growth [14]. Mills's studies [15] have shown that the rhamnosyl transferase from *Mycobacterium smegmatis* is essential for bacterial growth.

To determine whether RmlB and RmlC are essential enzymes of mycobacterial survival, we cloned a copy of *M. smegmatis* mc^{2}155 *rmlB* gene disrupted by a kanamycin resistance cassette orientated in the reversed direction from *rmlB* gene and selected a *M. smegmatis rmlB* gene knockout mutant strain. The ability of this mutant to synthesize the RmlB was dependent on the presence of a functional copy of the *M. tuberculosis rmlB* gene carried on a temperature-sensitive rescue plasmid. We demonstrate here that the mc^{2}155 *rmlB* gene knockout mutant is unable to grow at the higher temperature at which the rescue plasmid cannot be replicated. The downstream *rmlC* gene in mc^{2}155 *rmlB* gene knockout mutant would not be transcribed from the promoter of kanamycin resistance cassette due to a polar effect of *rmlB* mutation. Therefore, mc^{2}155 *rmlB* gene knockout mutant strain also requires the rescue plasmid carrying *M. tuberculosis rmlC* gene for its growth.

Through the construction and analysis of *M. smegmatis rmlB* knock out mutant, we demonstrate that both RmlB and RmlC are essential for mycobacterial growth. This work provides the direct evidence that the *M. tuberculosis rmlB* and *rmlC* genes involved in dTDP-rhamnose synthesis are valid targets for discovery of new anti-tuberculosis drugs.

Materials and methods

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* NovaBlue cells were grown in LB broth or LB agar 37 °C routinely. *M. smegmatis* mc^{2}155 strain was used for cloning *M. smegmatis rmlB* gene with its promoter region to construct a conditional replication plasmid as well as for allelic exchange experiments. *M. smegmatis* mc^{2}155 cells were grown in LB broth containing 0.05% Tween 80 or on LB agar at 37 °C routinely. Incubation of transformed mc^{2}155 cells with plasmids was at 30 and 42 °C depending on the experiment. Sucrose was added to the LB agar at final concentration of 10% when required.

When necessary, antibiotics were used at the following final concentrations: ampicillin (Amp), 100 μg/ml; kanamycin (Kan), 50 μg/ml (for NovaBlue) and 25 μg/ml (for mc^{2}155); gentamicin (Gen), 5 μg/ml, hygromycin (Hyg), 100 μg/ml (for NovaBlue) and 50 μg/ml (for mc^{2}155); and streptomycin (Str), 25 μg/ml (for NovaBlue) and 12.5 μg/ml (for mc^{2}155).

Preparation of M. smegmatis mc^{2}155 genomic DNA. The genomic DNA of *M. smegmatis* mc^{2}155 was prepared as described [16] with modification. mc^{2}155 cells from 5 ml culture were harvested by centrifugation at 4000*g* for 10 min, the cell pellet was resuspended in 250 μl solution I (25% sucrose, 50 mM Tris–HCl, pH 8.0, 50 mM EDTA, 500 μg/ml lysozyme, and 100 μg/ml RNase A) and incubated at 37 °C for 16 h. Two hundred and fifty-five microliters of solution II (100 mM Tris–HCl, pH 8.0, 1% SDS, and 400 μg/ml proteinase K) was added and the reaction mixture was incubated at 55 °C for 8 h. Genomic DNA was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1) and precipitated by 0.9 volume of isopropanol and 0.1 volume of 3 M NaAc. The DNA pellet was centrifuged at 12,000*g* for 20 min and washed by 70% ethanol. Genomic DNA was dissolved in 15 μl 1× TE buffer.

Construction of conditional replication plasmid carrying M. smegmatis rmlB::kan^R. The amino acid sequences of *M. tuberculosis* RmlB and RmlC proteins were obtained from *M. tuberculosis* H37Rv genome (http://genolist.pasteur.fr/TubercuList/). *M. tuberculosis* RmlB and RmlC were blasted against the genome of *M. smegmatis* mc^{2}155 and *rmlB* and *rmlC* homologs were found in one operon of mc^{2}155 genome. Sm rmlB1 primer (5′ CTCGGCGACAAGGTGCTCAAG 3′) and Sm rmlB2 primer (5′ TCACTGGCCACGCTCCTGGTAC 3′) were designed based on the *M. smegmatis rmB* and its upstream sequence and 1466 bp PCR product was amplified from mc^{2}155 genomic DNA by Vent DNA polymerase (New England Biolabs). The PCR product was purified with QIAEX II Gel Extraction Kit and then was cloned into the pSTBlue1 vector resulting in plasmid pYFM I (Table 1). The PCR product in pYFM I was sequenced using an ABI Prism 377 automated DNA sequencer (Applied Biosystems).

pYFM I was digested with *Sna*BI and *Hin*dIII to get *rmlB*, and *rmlB* was ligated to the *Hin*cII and *Hin*dIII sites of pBluescript II (KS+), yielding pYFM II. pUC4K was digested with *Bam*HI to get a kanamycin resistance cassette (*kan^R*, 1264 bp) which is originally from transposon

Table 1
Bacterial strains, plasmids, and probes used in this study

Strains/plasmids	Relevant characteristics	Source/reference
Bacteria		
E. coli NovaBlue	Used for cloning and plasmid propagation	Novagen
M. tuberculosis H37Rv	Pathogenic; used as a PCR template to amplify Tb *rmlB* and Tb *rmlC* genes	This study
M. smegmatis mc^{2}155	Wild type; non-pathogenic; used as a DNA template to amplify *rmlB* (996 bp) and its upstream sequence (470 bp) and used for allelic exchange experiments	ATCC
mc^{2}155 M1	*M. smegmatis* mc^{2}155 with pYFM IV integrated into the *rmlB* locus by pathway 1 (Fig. 2)	This study
mc^{2}155 M2-1	*M. smegmatis* mc^{2}155 M1 which has undergone a second crossover event in the presence of pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC*	This study
mc^{2}155 M2-2	*M. smegmatis* mc^{2}155 M1 which has undergone a second crossover event in the presence pCG76-Tb *rmlC* and pMVHG1-Tb *rmlB*. The genotype of mc^{2}155 M2-2 is identical to that of mc^{2}155 M2-1, except that they carry different rescue plasmids.	This study
Plasmids		
pSTBlue-1	Carries *amp^R* gene and *kan^R* gene; used for cloning PCR product with blunt ends	Novagen
pBluescript II (KS+)	Carries *amp^R* gene; used for cloning genes	Strategene
pMD18-T	Carries *amp^R* gene; used for cloning PCR product with A′ at 3′ ends	TaKaRa
pUC4K	Carries *amp^R* gene and kanamycin resistance cassette (*kan^R*)	Amersham Biosciences
pPR27	Carries *sacB* gene and *gen^R* gene; carries *E. coli* origin of replication and temperature-sensitive mycobacterial origin of replication.	[18]
pPR27-xylE	*xylE* gene from *Pseudomonas putida* was cloned to the *Bam*HI site of pPR27	This study
pYFM I	PCR product of *M. smegmatis rmlB* with its upstream sequence was cloned to the *Eco*RV site of pSTBlue 1	This study
pYFM II	*M. smegmatis rmlB* with its upstream sequence was cloned to the *Hin*cII and *Hin*dIII sites pBluescript II (KS+)	This study
pYFM III	*kan^R* was cloned to the *Sty*I site of *rmlB* in pYFM II	This study
pYFM IV	pPR27-xylE derivative carrying *rmlB*::*kan^R* (Fig. 2)	This study
pCG76	*E. coli*/*Mycobacterium* shuttle vector carrying a temperature-sensitive mycobacterial origin of replication, streptomycin resistance cassette (*str^R*)	[32]
pET23b-P$_{hsp60}$	Carries *amp^R* gene; *M. bovis* BCG hsp60 promoter was cloned to the *Xba*I and *Nde*I sites of pET23b	This study
pCG76-Tb *rmlB*	Temperature-sensitive rescue plasmid carrying the *M. tuberculosis rmlB* gene under control of BCG hsp60 promoter (Fig. 3)	This study
pCG76-Tb *rmlC*	Temperature-sensitive rescue plasmid carrying the *M. tuberculosis rmlC* gene under control of BCG hsp60 promoter (Fig. 3)	This study
pMVHG1	pVV16 derivative carrying *hyg^R* and BCG hsp60 promoter	[22]
pMVHG1-Tb *rmlB*	Rescue plasmid for Tb *rmlB* controlled by BCG hsp60 promote (Fig. 3)	This study
pMVHG1-Tb *rmlC*	Rescue plasmid for Tb *rmlC* controlled by BCG hsp60 promote (Fig. 3)	This study

Tn903 and encodes aminoglycoside 3′-phosphotransferase gene [17]. The kan^R fragment was filled in with Klenow and ligated to the *Sty*I site (filled in by Klenow) of pYFM II to generate pBluescript II-*rmlB*::kan^R (pYFM III). The kan^R has the reversed orientation with *rmlB* gene. The *rmlB*::kan^R fragment (2753 bp) was cut from pYFM III by *Eco*RI and both ends were filled in with Klenow, pPR27-xylE was digested with *Not*I and filled in with Klenow, and the *rmlB*::kan^R fragment was ligated to pPR27-xylE, yielding pPR27-xylE-*rmlB*::kan^R (pYFM IV) (Table 1, Fig. 2). pYFM IV is a conditional replication plasmid containing the mycobacterial temperature-sensitive origin of replication from the parent plasmid pPR27, thus it can replicate at 30 °C but is efficiently lost at 39 °C and above [18]. The single crossover event between *rmlB*::kan^R in pYFM IV and *rmlB* gene in the mc^2155 genome can happen at 39 °C and above, and integration of *rmlB*::kan^R into the mc^2155 genome generates a mc^2155 mutant strain, mc^2155 M1 (Table 1). Plasmid pYFM IV also harbors the counter-selectable marker *sacB* from *Bacillus subtilis* [19,20] for selecting mc^2155 *rmlB* gene knockout strain, mc^2155 M2 (Table 1) in the presence of sucrose.

Construction of rescue plasmids containing M. tuberculosis rmlB gene. pET16b-Tb *rmlB* [11] was digested with *Nde*I and *Xho*I to get Tb *rmlB*, and Tb *rmlB* was ligated to the *Nde*I and *Xho*I sites of pET23b-Phsp60 to generate pET23b-Phsp60-Tb *rmlB*. pET23b-Phsp60-Tb *rmlB* was digested with *Xho*I and treated with Klenow and the linear pET23b-Phsp60-Tb *rmlB* was digested with *Xba*I to get Phsp60-Tb *rmlB* fragment. pCG76 was digested with *Bam*HI and filled in by Klenow and the linear pCG76 was digested with *Xba*I. Phsp60-Tb *rmlB* fragment was ligated to pCG76, yielding pCG76-Tb *rmlB* (Table 1 and Fig. 3). The plasmid pCG76 carries the same temperature-sensitive mycobacterial replication origin as pPR27 and thus can replicate at the permissive temperature 30 °C but is lost at 39 °C and above [16]. pET16b-Tb *rmlB* was digested with *Nde*I and *Hin*dIII to get Tb *rmlB*, and Tb *rmlB* was ligated the *Nde*I and *Hin*dIII sites of pMHVG1 to generate pMHVG1-Tb *rmlB* (Table 1 and Fig. 3).

Construction of rescue plasmids containing M. tuberculosis rmlC gene. Primers as following were designed based on the DNA sequence of Tb *rmlC* gene: upstream primer with *Nde*I site, Tb rmlC1 (5′ CATA TGAAAGCACGCGAACTCG 3′) and downstream primer with *Bam*HI site, Tb rmlC2 (5′ GGATCCCTAGGTGCCGCGCATCT 3′). Tb *rmlC* was amplified from *M. tuberculosis* H37Rv genomic DNA by LA *Taq* DNA polymerase (Takara) and Tb *rmlC* PCR product was cloned to the *Eco*RV site of pMD18-T plasmid to generate pMD18-Tb *rmlC*. Tb *rmlC* PCR product was confirmed by sequencing. pMD18-Tb *rmlC* was digested with *Nde*I and *Bam*HI, Tb *rmlC* gene was ligated to the *Nde*I and *Bam*HI sites of pET23b-Phsp60 to generate pET23b-Phsp60-Tb *rmlC*. pET23b-Phsp60-Tb *rmlC* was digested by *Xba*I and *Bam*HI and the Phsp60-Tb *rmlC* fragment was ligated to the *Xba*I and *Bam*HI sites of pCG76 to generate pCG76-Tb *rmlC* (Table 1 and Fig. 3). pMD18-Tb *rmlC* was digested by *Nde*I and *Hin*dIII and Tb *rmlC* gene was ligated to the *Nde*I and *Hin*dIII sites of pMHVG1, yielding MHVG1-Tb *rmlC* (Table 1 and Fig. 3).

Preparation of DNA probe and Southern hybridization. The DNA probe was prepared by using DIG High Prime Labeling and Detection Starter Kit I (Roche). pYFM I was digested with *Not*I to get a 1261 bp DNA fragment containing 946 bp of *M. smegmatis rmlB* and 315 bp upstream sequence of *rmlB*. The DNA probe was labeled with digoxigenin by using 1261 bp DNA fragment as a template.

Mycobacterium smegmatis mc^2155 genomic DNA was digested overnight with *Sma*I and separated by electrophoresis through 0.7% agarose gel. The gel was treated with 0.25 M HCl for 10 min and then with 0.6 M

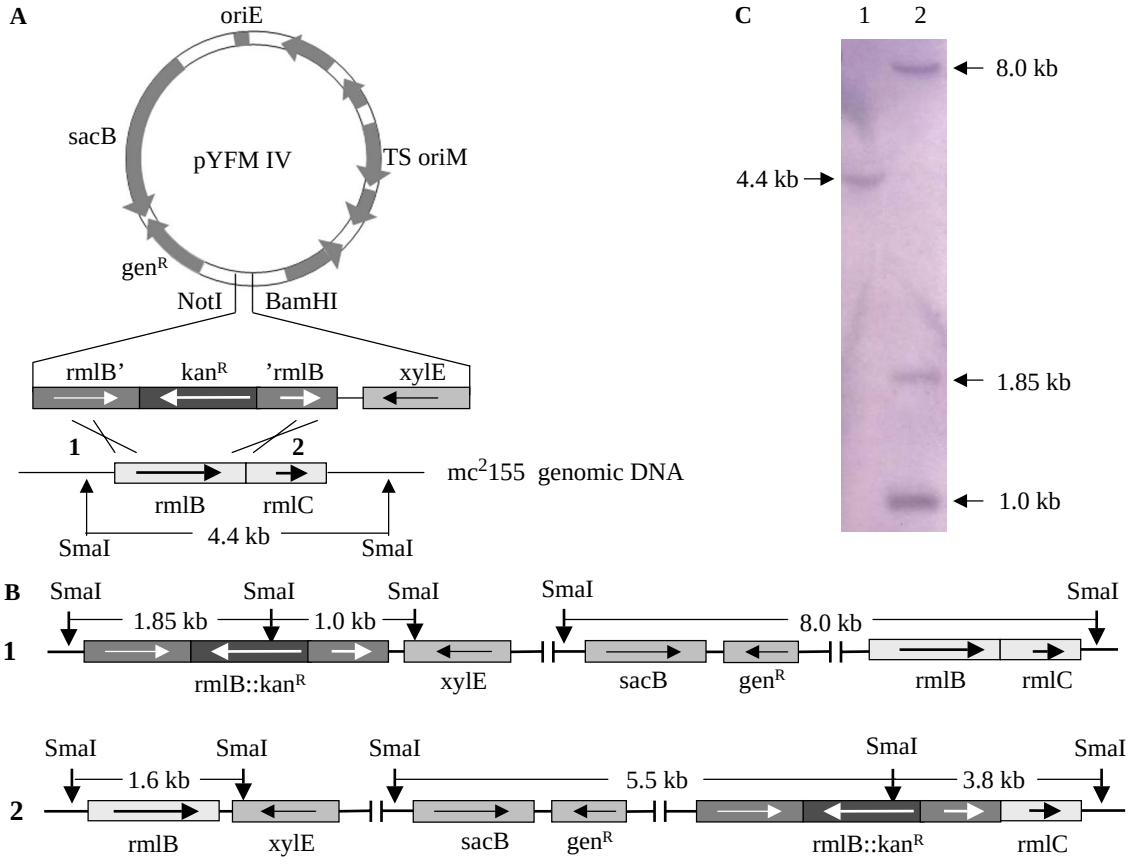

Fig. 2. (A) The map of pYFM IV. (B) Two possible pathways for homologous recombination between pYFM IV and the *M. smegmatis* mc^2155 genomic DNA. (C) Southern hybridization of *Sma*I-digested genomic DNA was used to confirm a single homologous recombination event at the *rmlB* locus of *M. smegmatis* mc^2155 genome. Lane 1, wild type of *M. smegmatis* mc^2155; lane 2, *M. smegmatis* mc^2155 M1 mutant resulted from pathway 1 homologous recombination.

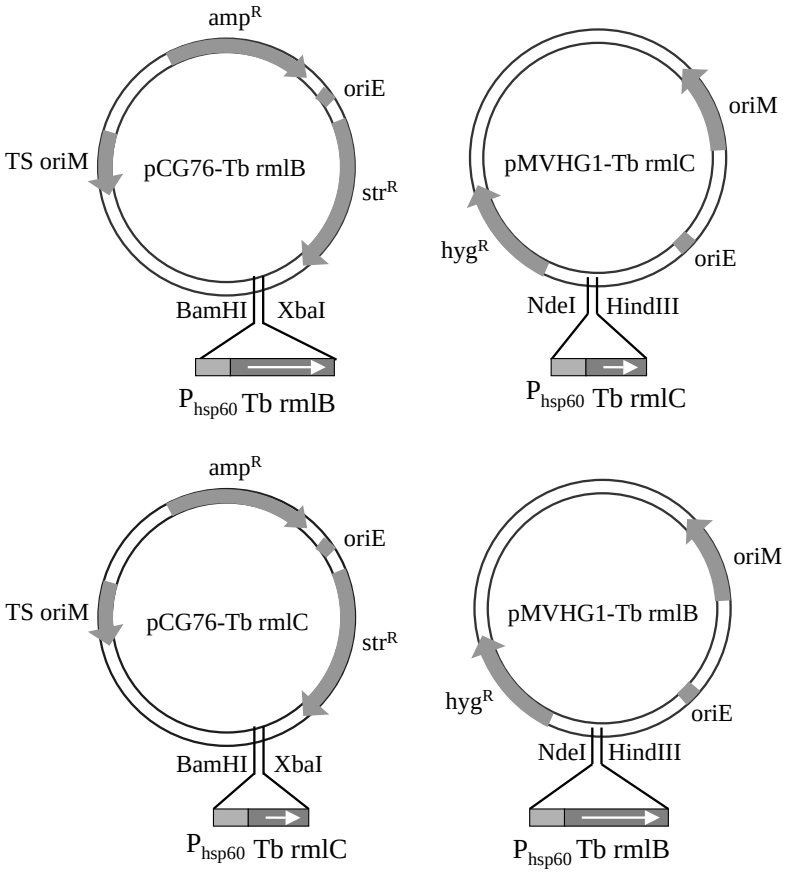

Fig. 3. Maps of pCG76-Tb *rmlB*, pMVHG1-Tb *rmlC*, pCG76-Tb *rmlC*, and pMVHG1-Tb *rmlB*. Expression of Tb *rmlB* and Tb *rmlC* was under control of the promoter of heat shock protein 60 from *M. bovis* BCG.

NaCl and 0.4 M NaOH for 30 min, and DNA was transferred in 0.6 M NaCl and 0.4 M NaOH to a positively charged Nytran membrane (Schleicher and Schuell). DNA hybridization and detection were performed according to Roche's protocol. The membrane was pre-hybridized at 42 °C for 1 h in DIG Easy Hyb and incubated with digoxigenin-labeled DNA probe at 42 °C for 16 h. The membrane was washed two times at room temperature with 2× SSC containing 0.1% SDS and then twice at 68 °C with 0.5× SSC containing 0.1% SDS. Colorimetric detection was carried out using DIG High Prime Labeling and Detection Starter Kit I (Roche).

Selection of mc^{2}155 M1 mutants carrying both rmlB and rmlB::kan^R. Electrocompetent mc^{2}155 was prepared as described [19], and pYFM IV was electroporated to mc^{2}155 cells by setting the voltage of Gene Pulser (Bio-Rad) to 2.5 kV, capacity to 25 μF, and resistance to 1000 Ω. Transformants were grown in LB agar plate containing Kan and Gen at 30 °C. One colony was inoculated into LB medium containing Kan and Gen and incubated at 30 °C. The cells were plated out onto LB agar plate containing Kan and Gen and incubated at 42 °C. mc^{2}155 M1 mutants with single homologous recombination were selected using Southern blot.

Selection of mc^{2}155 M2 mutants (rmlB gene knock out strains). pCG76-Tb *rmlB* and pMVGH1-Tb *rmlC* were electroporated to mc^{2}155 M1 mutant. Transformants were grown in LB agar plate containing Kan, Hyr, and Str and incubated at 30 °C. One colony was inoculated into LB medium containing Kan, Hyr, and Str and incubated at 30 °C. The cells were plated out onto LB agar plate containing 10% sucrose and Kan, Hyr, and Str. mc^{2}155 M2-1 mutants (Table 1) with double crossover were selected by using Southern blot. In a separate experiment, pCG76-Tb *rmlC* and pMVGH1-Tb *rmlB* were also electroporated to mc^{2}155 M1 mutant and mc^{2}155 M2-2 mutants (Table 1) were selected using Southern blot.

Growth of mc^{2}155 M2 mutants (rmlB gene knockout strains). *Mycobacterium smegmatis* mc^{2}155 M2-1 and mc^{2}155 M2-2 strains were inoculated in 5 ml of LB broth containing 0.05% Tween 80 and appropriate antibiotics, and the cells were incubated at both 30 and 42 °C. Transformed mc^{2}155 M1 with pCG76-Tb *rmlB* and pMVGH1-Tb *rmlC* as a control. The absorption at wavelength of 600 nm was measured at interval of 24 h and the growth curves of mc^{2}155 M2-1 and mc^{2}155 M2-2 were obtained.

Results

Construction of conditional replication plasmid carrying M. smegmatis rmlB::kan^R

The PCR product (1466 bp) amplified from *M. smegmatis* mc^{2}155 genomic DNA was cloned to pSTBlue1 plasmid. Sequences of cloned PCR product contained the entire *rmlB* gene (996 bp) and 470 bp upstream sequence from the start codon (ATG) of *rmlB* gene, and this 470 bp DNA sequence thus presumably included *rmlB* promoter.

M. smegmatis rmlB gene had a unique *Sty*I site in the 449 bp of *rmlB* into which a kanamycin resistance cassette (*kan^R*) was inserted and shown to be oriented in the reversed direction as *rmlB* by restriction enzyme digests. This was the desired orientation of the *kan^R* gene as it was hoped that this orientation would result in polar effect on downstream *rmlC* gene. The *rmlB*::*kan^R* fragment was inserted into pPR27-*xylE* to yield pYFM IV (Fig. 2A). pPR27 plasmid carries the mutated origin of mycobacterial

replication and replicates in mycobacteria only at the permissive temperature (30 °C) but not at the non-permissive temperature (42 °C). Therefore, pYFM IV derived from pPR27 facilitates obtaining recombinant mc^{2}155 strains that have undergone a single homologous recombination event at the *rmlB* locus by selecting pYFM IV-transformed mc^{2}155 at 42 °C. The colored marker *xylE* gene [21] in pYFM IV is utilized to screen the mc^{2}155 mutants that contain both *rmlB* gene and *rmlB*::*kan*^{R} mutation gene, and the counter-selectable marker *sacB* gene [19,20] in pYFM IV facilitates obtaining the mc^{2}155 mutants that contain only *rmlB*::*kan*^{R} mutation gene (i.e., *rmlB* gene knocked out).

Selection of mc^{2}155 M1 mutants carrying both rmlB and rmlB::kan^R

The plasmid pYFM IV was electroporated into *M. smegmatis* mc^{2}155 and transformed mc^{2}155 cells were selected on LB agar containing Kan at 30 °C. One transformant was then propagated in LB medium containing Kan and Gen at 30 °C and then plated out on LB agar plate containing Kan and Gen at 42 °C. Since the temperature-sensitive plasmid pYFM IV is able to replicate at 30 °C but not at 42 °C the kanamycin resistant colonies that appear on LB agar containing Kan have necessarily integrated the *kan*^{R} gene into their genome. All colonies turned to yellow when sprayed with catechol because all colonies expressed *xylE* gene. Single homologous recombination between *rmlB* gene in the genome and *rmlB*::*kan*^{R} in pYFM I may occur upstream and downstream from *kan*^{R} gene resulting in genotypes 1 and 2 are shown in Fig. 2B. Illegitimate recombination may also occur which would leave *rmlB* and *rmlC* fully intact.

*Sma*I-digested genomic DNA from 16 yellow colonies grown at 42 °C was analyzed by Southern hybridization of *rmlB* probe. The results have shown that 1 colony came from homologous recombination pathway 1 containing an intact *rmlB* containing operon (Fig. 2C) and 15 colonies came from illegitimate recombination (data not shown). The colony with homologous recombination pathway 1 was named mc^{2}155 M1 (Table 1) and was propagated for further experiments. Since *kan*^{R} gene was inserted into *rmlB* gene at the inverted orientation, the *rmlC* gene at the downstream of *rmlB* gene would not be transcribed by the natural promoter of *rmlB* or the promoter of *kan*^{R} gene, therefore, no colonies came from homologous recombination pathway 2 were selected. No colonies with double crossover event that *rmlB*::*kan*^{R} replaced *rmlB* gene were selected when the rescue plasmids carrying *rmlB* and *rmlC* genes were not present. These results suggested that both *rmlB* and *rmlC* are essential genes.

Construction of rescue plasmids

To attempt the second homologous recombination event two sets of rescue plasmids carrying *M. tuberculosis rmlB* and *rmlC* genes were constructed. The first set of rescue plasmids consisted of pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC* (Table 1 and Fig. 3) and the second set of rescue plasmids consisted of pCG76-Tb *rmlC* and pMVHG1-Tb *rmlB* (Table 1 and Fig. 3). The expression of both *M. tuberculosis rmlB* and *rmlC* genes was under control of the promoter of *Mycobacterium bovis* BCG heat shock protein 60 (P_{hsp60}) [22,23]. pCG76-Tb *rmlB* and pCG76-Tb *rmlC* were derived from pCG76, the same temperature-sensitive origin of replication used in pYFM IV so that the essentiality of Tb *rmlB* and Tb *rmlC* gene in mc^{2}155 M2 mutants could be tested.

Selection of mc^{2}155 M2 mutants (rmlB gene knockout strains)

Single colony of mc^{2}155 M1 with rescue plasmids was grown in LB medium containing Kan, Str, Hyg, and 0.05% Tween 80 at 30 °C and then plated onto LB agar plates containing Kan, Str, Hyg, and 10% sucrose at 30 °C. Analyses of XylE phenotype of resulting colonies showed that 65% colonies with pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC* were "white" color and 100% colonies with pCG76-Tb *rmlC* and pMVHG1-Tb *rmlB* were "white" color. The colonies that have undergone a second crossover should both be able to grow on sucrose and have lost the XylE marker; colonies that can grow on sucrose but still express *xylE* are likely to be *sacB* mutants rather than arising from the second crossover event. Thus, only the "white" colonies are candidates for the second crossover event occurring. Eleven of the white colonies carrying pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC* were analyzed by *Sma*I digestion and Southern blot; all eleven showed bands at 1.85, 3.82, and 6.0 kb as expected (Fig. 4B) for the second single crossover event (the 6.0 kb band is from rescue plasmid pCG76-Tb *rmlB*). These strains were named mc^{2}155 M2-1 (Table 1). Fifteen of the white colonies carrying pCG76-Tb *rmlC* and pMVHG1-Tb *rmlB* were analyzed by *Sma*I digestion and Southern blot; all 15 showed bands at 1.85, 3.82, and 5.8 kb as expected (Fig. 4C) for the second single crossover event (the 5.8 kb band is from rescue plasmid pMVHG1-Tb *rmlB*). These strains were named mc^{2}155 M2-2 (Table 1). mc^{2}155 M2-1 and mc^{2}155 M2-2 have the same genotype even though they carry different rescue plasmids. Three colonies of mc^{2}155 M2-1 and mc^{2}155 M2-2 were propagated for monitoring growth curve.

Mycobacterium smegmatis mc^{2}155 M2 will not grow at 42 °C

As final experiments to confirm that RmlB (dTDP-D-glucose-4,6-dehydratase) and RmlC (dTDP-4-keto-6-deoxyglucose-3,5-epimerase) are essential for growth, growth curves at 30 and 42 °C were obtained (Fig. 5) for mc^{2}155 M2-1 and mc^{2}155 M2-2 and mc^{2}155 M1 containing pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC*. The results clearly showed that mc^{2}155 M2-1 and mc^{2}155 M2-2 grew at

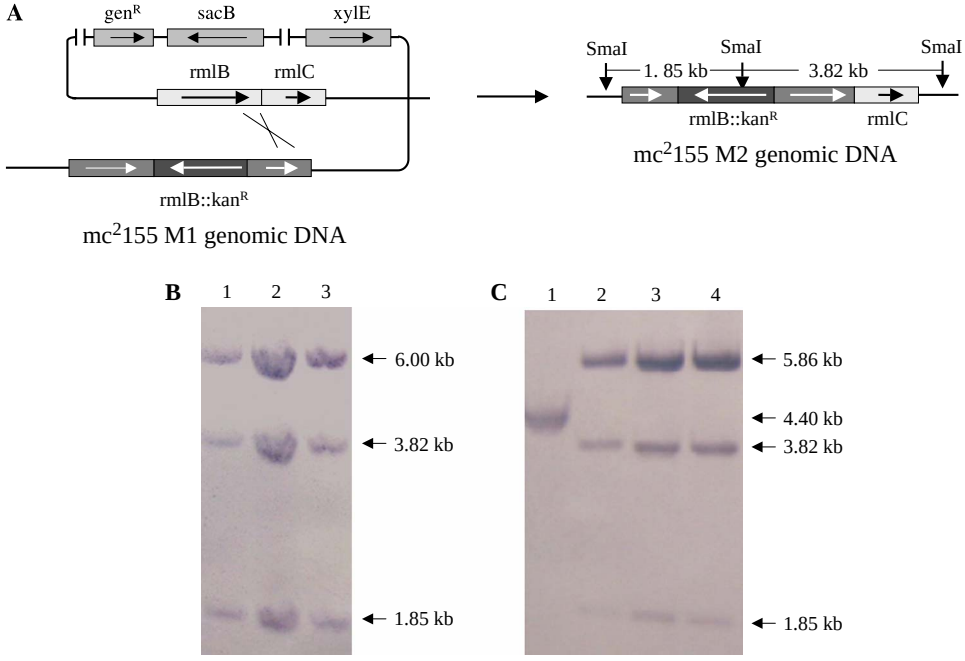

Fig. 4. (A) The second crossover event resulting in *rmlB* knockout which occurs in the presence of rescue plasmids carrying Tb *rmlB* and Tb *rmlC* gene, respectively. (B) Southern hybridization of *Sma*I-digested genomic DNA was used to confirm of *M. smegmatis* mc^{2}155 M2-1 mutant (*rmlB* knockout strain). Lanes 1–3, three mc^{2}155 M2-1 mutants containing pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC* rescue plasmids generate 1.85, 3.82, and 6.0 kb fragments. The fragment of 6.0 kb comes from the rescue plasmid pCG76-Tb rmlB. (C) Southern hybridization of *Sma*I-digested genomic DNA was used to confirm of *M. smegmatis* mc^{2}155 M2-2 mutant (*rmlB* knockout strain). Lane 1, wild type of mc^{2}155 shows 4.4 kb fragment; lanes 2–4, three mc^{2}155 M2-2 containing pCG76-Tb *rmlC* and pMVHG1-Tb *rmlB* rescue plasmids generate 1.85, 3.82, and 5.86 kb fragments. The fragment of 5.86 kb comes from the rescue plasmid pMVGH1-Tb *rmlB*. The genotype of mc^{2}155 M2-1 mutants and mc^{2}155 M2-2 mutants are identical.

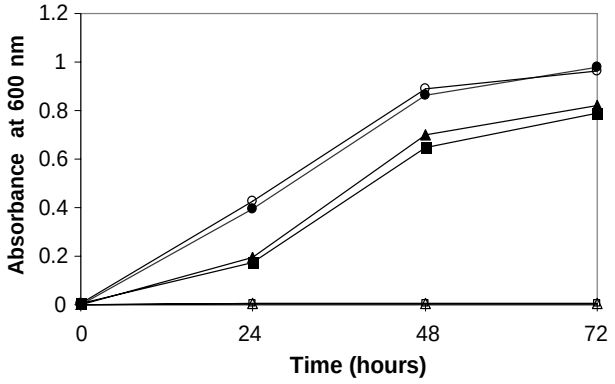

Fig. 5. Growth curves of *M. smegmatis* strains at 30 and 42 °C. *M. smegmatis* mc^{2}155 M2-1 containing rescue plasmids pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC* (■) and mc^{2}155 M2-2 containing rescue plasmids pCG76-Tb *rmlC* and pMVHG1-Tb *rmlB* (▲) at 30 °C; *M. smegmatis* mc^{2}155 M2-1 containing rescue plasmids pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC* (□) and mc^{2}155 M2-2 containing rescue plasmids pCG76-Tb *rmlC* and pMVHG1-Tb *rmlB* (△) at 42 °C; *M. smegmatis* mc^{2}155 M1 containing rescue plasmids pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC* at 30 °C (●); mc^{2}155 M1 containing rescue plasmids pCG76-Tb *rmlB* and pMVHG1-Tb *rmlC* at 42 °C (○).

30 °C, but did not grow at 42 °C at which the rescue plasmids (pCG76-Tb *rmlB* and pCG76-Tb *rmlC*) were unable to replicate. Therefore, the results confirmed that both Tb *rmlB* and Tb *rmlC* genes were essential for mycobacterial growth.

Discussion

Tuberculosis (TB) remains a major cause of morbidity and mortality throughout the world, therefore, it is urgent to develop more new anti-tuberculosis drugs. The arabinogalactan of mycobacterial cell wall is potential drug target because of its unique structure. The sugar donor formation enzymes and sugar transferases involved in arabinogalactan biosynthesis have been identified so far [11,24–27]. Some of them have been identified as essential drug target by genetic knockouts [14,28]. The microtiter based assays of *M. tuberculosis* Glf enzyme [29] and *M. tuberculosis* RmlB-D enzymes [11] have been developed to screen inhibitors for Glf and RmlB-D. It is clear that *M. tuberculosis* RmlD is essential for the growth of *M. smegmatis rmlD* gene knockout strains [14]. The goal of this study was to investigate the essentiality of RmlB and RmlC for mycobacterial survival.

The model organism *M. smegmatis* mc^{2}155 used in generating gene knockout strains has many advantages including low biohazard risk, rapid growth, ability to grow on simple media, high efficient transformability, and availability of a temperature-sensitive replication origin of plasmid for it. All mycobacteria including *M. tuberculosis* and *M. smegmatis* have the basic structure of the cell wall [30] and the exact same linker structure with the identically positioned rhamnosyl residue. BLAST analysis shows that *rmlA-D* genes of *M. smegmatis* mc^{2}155 are in three various

operons in the genome and *rmlB* and *rmlC* genes are together as in *M. tuberculosis* H37Rv genome. Therefore, we constructed a conditional replication plasmid carrying *M. smegmatis rmlB* gene where kanamycin resistance cassette was inserted at the reversed orientation as *rmlB* gene. It might result in the generation of a polar effect of *rmlB* gene knockout, thus the downstream *rmlC* gene would not be transcribed from the promoter of *rmlB* gene, that is, expression of *rmlC* gene presumably would be affected. Although it cannot yet be generalized that inserting the kanamycin resistance cassette in the same orientation of the gene to be knocked out will result in a non-polar knockout and that inserting the cassette in the opposite direction to the coding direction will result in a polar mutation, the genetic knockouts of *M. smegmatis glf* gene [28,31] and *M. smegmatis rmlD* gene [14] provide the evidences. *M. smegmatis* galactopyranose mutase gene (*glf*) was knocked out with the kanamycin resistance cassette oriented in the opposite direction to the coding direction of *glf* and the mutation was indeed polar as the gene downstream of *glf*, Rv3808c, was not transcribed. Inserting the kanamycin resistance cassette in the coding direction of *M. smegmatis rmlD* gene did indeed result in a non-polar mutation and the downstream gene, rhamnosyl transferase gene (*wbbL*) was transcribed which may from the promoter of kanamycin resistance gene.

There are three rmlBs (*rmlB*, *rmlB2*, and *rmlB3*) in *M. tuberculosis* H37Rv genome [10]. Ma's data [11] show that expressed *M. tuberculosis rmlB* (Rv3464) gene product has dTDP-D-glucose 4,6-dehydratase activity, but the protein encoded by *M. tuberculosis rmlB3* (Rv3468c) gene does not have dTDP-D-glucose 4,6-dehydratase activity. Though the protein encoded by *M. tuberculosis rmlB2* (Rv3634c) gene has homology to dTDP-D-glucose 4,6-dehydratase, but its N-terminal sequence is nearly identical to that of UDP-galactose epimerase of *M. smegmatis* [24], therefore, the *rmlB2* gene product should be UDP-galactose epimerase. In this genetic knockout study, we found that (1) no colonies with double crossover event (*rmlB*::kan^R replacing *rmlB* gene) were selected when *M. smegmatis* mc^2155 was transformed by conditional replication plasmid pYFM IV; (2) *rmlB* gene was knocked out in *M. smegmatis* mc^2155 genome only when the rescue plasmids carrying *M. tuberculosis rmlB* and *rmlC* genes were present; (3) mc^2155 M2 mutant strains were grown only when *M. tuberculosis rmlB* and *rmlC* genes in rescue plasmids were expressed at 30 °C. These genetic results demonstrate that only one *rmlB* and *rmlC* gene copy exists in *M. smegmatis* genome, and *rmlB* and *rmlC* are essential genes for growth of *M. smegmatis*. Thus, our studies support the further exploration of RmlB and RmlC as targets for mycobacterial-drug development.

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