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NOTES

Formation of dTDP-Rhamnose Is Essential for Growth of Mycobacteria

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It was determined that the dTDP-rhamnose synthesis gene, *rmlD*, could be inactivated in *Mycobacterium smegmatis* only in the presence of a rescue plasmid carrying functional *rmlD*. Hence, dTDP-rhamnose biosynthesis is essential for the growth of mycobacteria and the targeting of dTDP-rhamnose synthesis for new tuberculosis drugs is supported.

The mycobacterial cell wall consists of a mycolic acid layer tethered to peptidoglycan via the polysaccharide arabinogalactan (3, 13, 14). Arbinogalactan is attached to peptidoglycan via α-L-rhamnopyranosyl-(1→3)-α-D-*N*-acetyglucosaminosyl-1-phosphate (13). This structural arrangement strongly suggests that rhamnosyl residues are essential for the growth and viability of mycobacteria.

L-Rhamnosyl residues are not present in humans. They are synthesized in bacteria from dTDP-glucose via the enzymes RmlB, RmlC, and RmlD (5, 8, 15, 17). There is no salvage pathway for the formation of dTDP-L-rhamnose (dTDP-Rha) as there is with GDP-L-fucose (19, 20). The dTDP-Rha synthetic enzymes are soluble and have been studied by X-ray crystallography (1, 6, 7). Given these facts, the dTDP-Rha formation enzymes have significant potential as targets for new tuberculosis drugs. Therefore, it is important to directly demonstrate that an enzyme involved in the formation of these enzymes is essential.

The final enzyme in the series synthesizing dTDP-Rha is dTDP-6-deoxy-L-*lyxo*-4-hexulose reductase. Its gene, *rmlD*, is the first gene of an operon which also contains *wbbL* and *manB* (12). This arrangement occurs in all the mycobacterial genomes sequenced. The gene *wbbL* encodes the rhamnosyl transferase that inserts rhamnose into the cell wall and is also expected to be essential. Thus, a nonpolar *rmlD* knockout mutation was desired so that complementation would be straightforward.

The basic strategy used was to prepare a copy of *rmlD* interrupted with a kanamycin resistance cassette orientated in the same direction as *rmlD* with the hope that, after gene replacement, the downstream *wbbL* and *manB* genes would be transcribed from the kanamycin resistance promoter. The disrupted *rmlD* gene was then used to replace wild-type *rmlD* in the presence of an appropriate rescue plasmid.

Constructing the *rmlD* replacement plasmid (pFP201) and obtaining the first homologous recombination event. A partial genomic DNA library of a mycobacterial lab strain (18) was constructed by isolation of *Sma*I fragments of approximately 3.5 kb and ligation into pCR-Blunt (Invitrogen, Carlsbad, Calif.). The *rmlD* gene was located by colony hybridization (9) using *Mycobacterium tuberculosis rmlD* (10) as a probe. Preliminary sequence data showed that the *rmlD* DNA sequence was almost identical to that of *Mycobacterium smegmatis* mc^2155 (The Institute for Genomic Research website [http://www.tigr.org/]). Then a 1.6-kb DNA fragment (containing *rmlD* and 417 bp upstream and 328 bp downstream of it) was cut out by *Eco*RI and *Xho*I, filled in with the Klenow fragment, and then inserted into pCR-Blunt to generate pCR-*rmlD*. By using methods previously described (18), pCR-*rmlD* was used to construct pFP201 (Table 1), a plasmid with a temperature-sensitive (TS) origin of replication which carries *rmlD*::*kan*, *sacB*, and *xylE*. Although the orientation of *kan* in pFP201 was not controlled by the procedure used (18), it was shown in the case of pFP201 to be the same as that of *rmlD* by restriction enzyme digestion. Plasmid pFP201 was then electroporated into *M. smegmatis* mc^2155, and a transformant was propagated in Luria-Bertani broth (LB)–kanamycin medium at 30°C, followed by plating onto LB-kanamycin plates at 42°C. Since the TS plasmid was able to replicate at 30°C but not at 42°C, the kanamycin-resistant colonies that appeared on the 42°C plates had necessarily integrated the *kan* gene into their chromosome. Analysis of colonies on these plates by Southern blotting revealed a colony arising from homologous recombination (Fig. 1) which was propagated for further experiments (*M. smegmatis* FP201) (Table 1).

Construction of an *rmlD* rescue plasmid. A rescue plasmid, pYM201 (Table 1), was constructed by digesting the *M. tuberculosis* bacterial artificial chromosome Rv3 clone (2) with *Not*I and *Avr*-II, yielding a 3,747-bp fragment containing the *rmlD-wbbL-manB* operon. The DNA ends were filled in (End Conversion Mix; Novagen, Madison, Wis.) and ligated to pST-Blue-1 (Novagen) to generate the pSTB1-*M. tuberculosis rmlD* operon. This plasmid was digested with *Eco*RI and *Xba*I to get a 1.5-kb fragment containing *rmlD* and approximately 550 bp

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TABLE 1. Key bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
M. smegmatis mc^2 155	Harbors all plasmids used herein	22
M. smegmatis FP201	*M. smegmatis* mc^2 155 with pFP201 integrated into the *rmlD* locus (Fig. 1)	This study
M. smegmatis YM202	*M. smegmatis* FP201 which has undergone a second-crossover event in the presence of pYM201 (Fig. 1)	This study
Plasmids		
pPR27	TS mycobacterial origin of replication; carries *sacB* gene, *gen*, *E. coli* origin of replication	21
pFP201	pPR27 derivative carrying *rmlD*::*kan* and the *xylE* gene	This study
pCG76	*Escherichia coli*/*Mycobacterium* shuttle vector carrying a TS mycobacterial origin of replication and streptomycin resistance cassette (*str*)	11
pYM201	TS rescue plasmid pCG76 carrying the *M. tuberculosis rmlD* gene under the control of the *rmlD* natural promoter, *str*	This study

of DNA upstream of the start codon. The fragment was filled in with the Klenow fragment and ligated to *Xba*I-cleaved and -blunted pCG76 (18) to generate pYM201 (Table 1). The plasmid had the same TS origin of replication used in pFP201.

Second-crossover attempts and events. Single-colony isolates of *M. smegmatis* FP201 with and without rescue plasmid were grown in LB-kanamycin medium at 30°C and then plated onto LB-kanamycin-sucrose plates at 30°C. The resulting col-

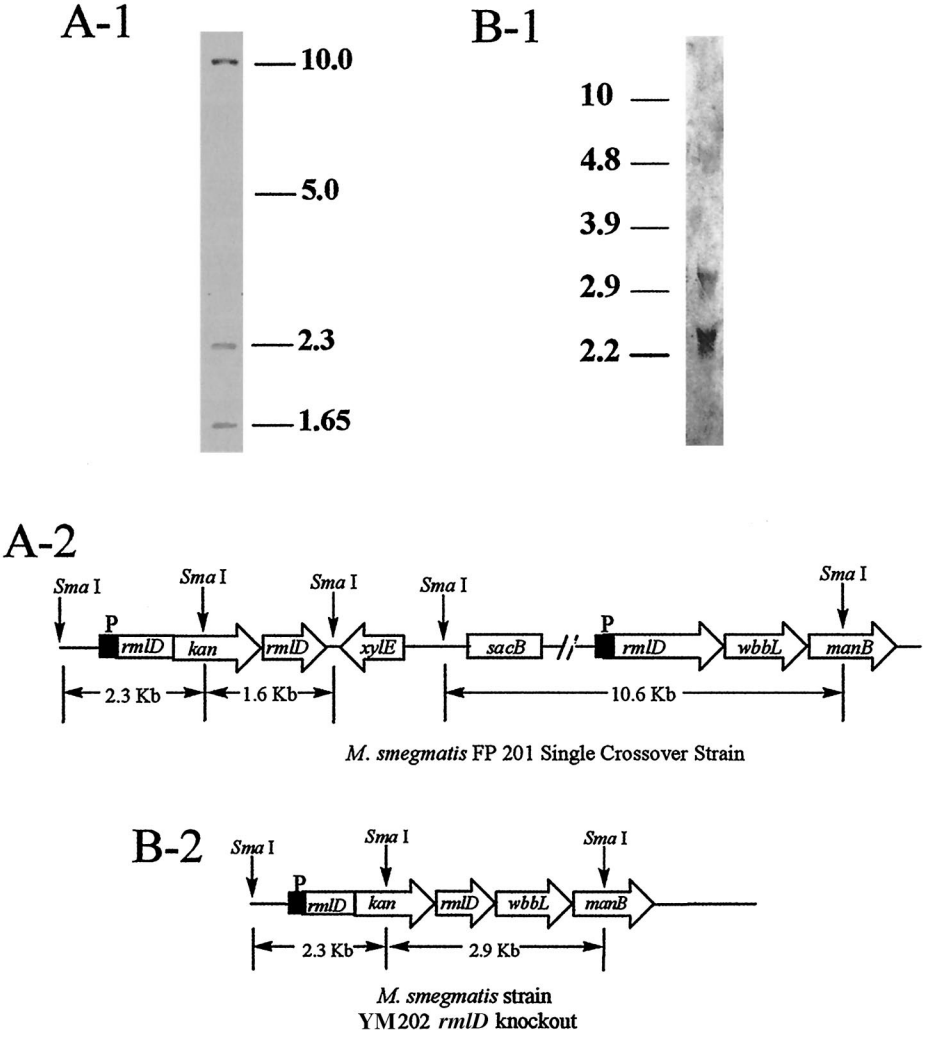

FIG. 1. Southern blot analysis of *M. smegmatis.* (A-1) Southern blot of DNA from the first-crossover strain, *M. smegmatis* FP201 (Table 1). DNA was cleaved with *Sma*I, and the probe was *rmlD.* (A-2) Origins of the fragments. (B-1) Southern blot of DNA from the second-crossover strain, *M. smegmatis* YM202 (Table 1). DNA was cleaved with *Sma*I, and the probe was *rmlD.* (B-2) Origins of the fragments. In the original blot, a fragment at 10 kb that originated from the rescue plasmid was faintly visible. Values to the sides of the blots are molecular sizes (in kilobases).

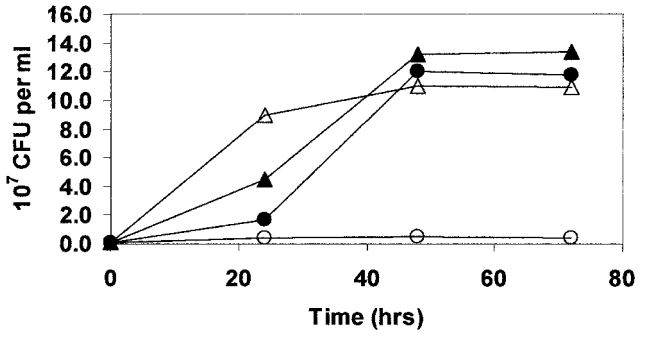

FIG. 2. Growth curves of *M. smegmatis* strains at 30 and 42°C. The growth of the control strain, *M. smegmatis* mc^{2}155, containing plasmid pYM201 at 30°C (▲) and at 42°C (△), and the growth of the *rmlD* knockout strain, *M. smegmatis* YM202, containing plasmid pYM201 at 30°C (●) and at 42°C (○), are illustrated.

onies were analyzed for their XylE phenotype (a yellow color develops in colonies expressing *xylE* when they are sprayed with catechol). Colonies that have undergone a second crossover should both be able to grow on sucrose and have lost the capacity for XylE enzyme production; colonies that can grow on sucrose but still express *xylE* are likely to arise from mutations in *sacB* rather than from the second-crossover event. Examination of the data revealed that only yellow colonies were obtained without the rescued plasmid but that 51% of the colonies were white (*xylE* absent) and 49% of the colonies were yellow when the rescue plasmid was present. Eighteen of these white colonies were analyzed by *Sma*I digestion and Southern blot analysis; all 18 showed bands at 2.3, 2.9, and 10.6 kb (the 10.6-kb band was from the rescue plasmid) as expected (Fig. 1) for the second single-crossover event. One colony, designated *M. smegmatis* YM202 (Table 1), was propagated for further experiments.

***M. smegmatis* YM202 will not grow at 42°C.** Curves indicating growth at 30 and 42°C were obtained (Fig. 2) for *M. smegmatis* YM202 containing the rescue plasmid pYM201 and, as a control, for wild-type *M. smegmatis* mc^{2}155 containing pYM201. The results clearly showed that *M. smegmatis* YM202 was unable to grow at the temperature at which the rescue plasmid was lost, confirming that *rmlD* is essential for growth.

The *wbbL* gene is transcribed and translated in YM202. To confirm transcription of *wbbL*, we prepared mRNA from *M. smegmatis* YM202 and showed that it hybridized with the *wbbL* DNA probe (data not shown). To confirm translation, we ran an enzyme assay for rhamnosyl transferase (16) and showed by thin-layer chromatography that the product of WbbL, α-L-Rha-(1→3)-α-D-GlcNAc-(1→P)-P-decaprenyl, was formed by membranes prepared from *M. smegmatis* YM202 (data not shown). Thus, the knockout is clearly a nonpolar event. In an earlier study (18), where the galactopyranose mutase gene (*glf*) was knocked out with the kanamycin cassette oriented in the direction opposite to the coding direction of *glf*, the mutation was polar since the gene downstream of *glf*, *Rv3808c*, was not transcribed. (In the original publication, the orientation of the kanamycin cassette was presented as being in the same direction as *glf*. However, the assignment was in error and an author correction to that effect has been published [see reference 18]).

Application of results to *M. tuberculosis*. The experiments were done with *M. smegmatis* due to the fast-growth characteristics of this organism and the availability of a TS origin of replication for it. We have shown that the basic structures of the cell walls of all mycobacteria are indistinguishable from one another by ^{13}C nuclear magnetic resonance and oligosaccharide profiling (4) and, in particular, that all mycobacteria have exactly the same linker structure with an identically positioned rhamnosyl residue (13). In addition, *rmlA*, *rmlB*, *rmlC*, and *rmlD* are found in the genomes of *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium*, *M. smegmatis*, and *Mycobacterium leprae* (12). Thus, the identity of the rhamnosyl-containing linker, along with the genetics, strongly argues that dTDP-Rha formation enzymes are essential in all mycobacteria, including *M. tuberculosis*.

It is worthwhile to note that an understanding of the unique structural role of the L-rhamnosyl residue in the cell walls of *M. tuberculosis* cells (13) was required before RmlD emerged as an important drug target; such an insight is not available from the genome sequence alone and underscores the fact that fundamental organism-specific biochemistry is required to most effectively exploit genome data. The expression of *rmlD* (10) and development of microtiter-based assays to detect inhibitors of the Rml enzymes (12) have also been accomplished. With these assays, X-ray crystallographic studies (7), and the work reported herein, the framework to proceed in the development of drugs against dTDP-rhamnose synthesis is in place.

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