

Analysis of the Precursor rRNA Fractions of Rapidly Growing Mycobacteria: Quantification by Methods That Include the Use of a Promoter (*rrnA* P1) as a Novel Standard†

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Mycobacterial species are able to control rRNA production through variations in the number and strength of promoters controlling their *rrn* operons. *Mycobacterium chelonae* and *M. fortuitum* are members of the rapidly growing mycobacterial group. They carry a total of five promoters each, encoded, respectively, by one and two *rrn* operons per genome. Quantification of precursor *rrn* transcriptional products (pre-*rrn*) has allowed detection of different promoter usage during cell growth. Bacteria growing in several culture media with different nutrient contents were compared. Balanced to stationary phases were analyzed. Most promoters were found to be used at different levels depending on the stage of bacterial growth and the nutrient content of the culture medium. Some biological implications are discussed. Sequences of the several promoters showed motifs that could be correlated to their particular level of usage. A product corresponding to the first *rrnA* promoter in both species, namely, *rrnA* P1, was found to contribute at a low and near-constant level to pre-rRNA synthesis, regardless of the culture medium used and the stage of growth analyzed. This product was used as a standard to quantitate rRNA gene expression by real-time PCR when *M. fortuitum* infected macrophages. It was shown that this bacterium actively synthesizes rRNA during the course of infection and that one of its *rrn* operons is preferentially used under such conditions.

It is generally assumed that, to satisfy the cell's demand for protein synthesis, the number of ribosomes per cell is proportional to the growth rate. This phenomenon has been termed growth rate-dependent control of ribosome synthesis (for a review, see reference 22). Protein synthesis and RNA content have been shown to be correlated both in faster-growing bacteria, such as *Escherichia coli*, and in slower-growing bacteria, such as *Mycobacterium bovis* BCG (7).

Mycobacterium is considered a genus with a slow growth rate compared to those of other human-pathogenic bacteria such as *E. coli*. Mycobacteria have a minimum number (one or two) of *rrn* operons per genome (2, 9).

The *rrn* operons of mycobacteria are members of the *rrnA* and *rrnB* families (15). The single *rrn* operons of several mycobacterial species, such as *Mycobacterium tuberculosis* and *M. avium*, belong to the *rrnA* family. rRNA operons that are members of the *rrnA* family have characteristic features: they are controlled by two or more tandem promoters, and they are located within the genome downstream from the *murA* gene. This type of *rrn* operon is present in all the mycobacterial species analyzed thus far. Members of the *rrnB* family also have

characteristic features: they are controlled by a single promoter and are located in the genome downstream from the *tyrS* gene (20).

A strategy developed by mycobacteria to control their rRNA synthesis is apparently based on variation in the number and strength of promoters controlling transcription. Mycobacterial species that grow very slowly, such as *M. tuberculosis*, have two promoters controlling their rRNA synthesis, but those species growing faster, such as *M. smegmatis*, have four promoters (13).

It has been shown that *M. smegmatis* varies its amount of rRNA at different stages of the growth curve; the level is greater when bacteria are growing rapidly in a rich nutrient medium, and it decreases to a lower level as *M. smegmatis* grows slowly, e.g., in stationary phase in a rich nutrient medium or when growing in a limited-carbon-source medium. The amount of rRNA was maintained at a low level in *M. tuberculosis*, regardless of whether *M. tuberculosis* was at the log or the stationary phase (14).

A possible way by which mycobacteria can control the rRNA production is through variations in the use of their different *rrn* promoters. Primer extension (PE) is a quantitative method that has been used to map promoters and to describe differences in *rrn* promoter usage in both *M. tuberculosis* and *M. smegmatis* (14), which have, respectively, a single *rrn* operon and 2 *rrn* operons per genome (2). *M. tuberculosis* has two promoters driving its single *rrnA* operon, one of which was used 2 to 4 times more efficiently than the other at all stages of growth tested (14, 26). *M. smegmatis* has a total of four promoters, three driving *rrnA* operon synthesis and a single pro-

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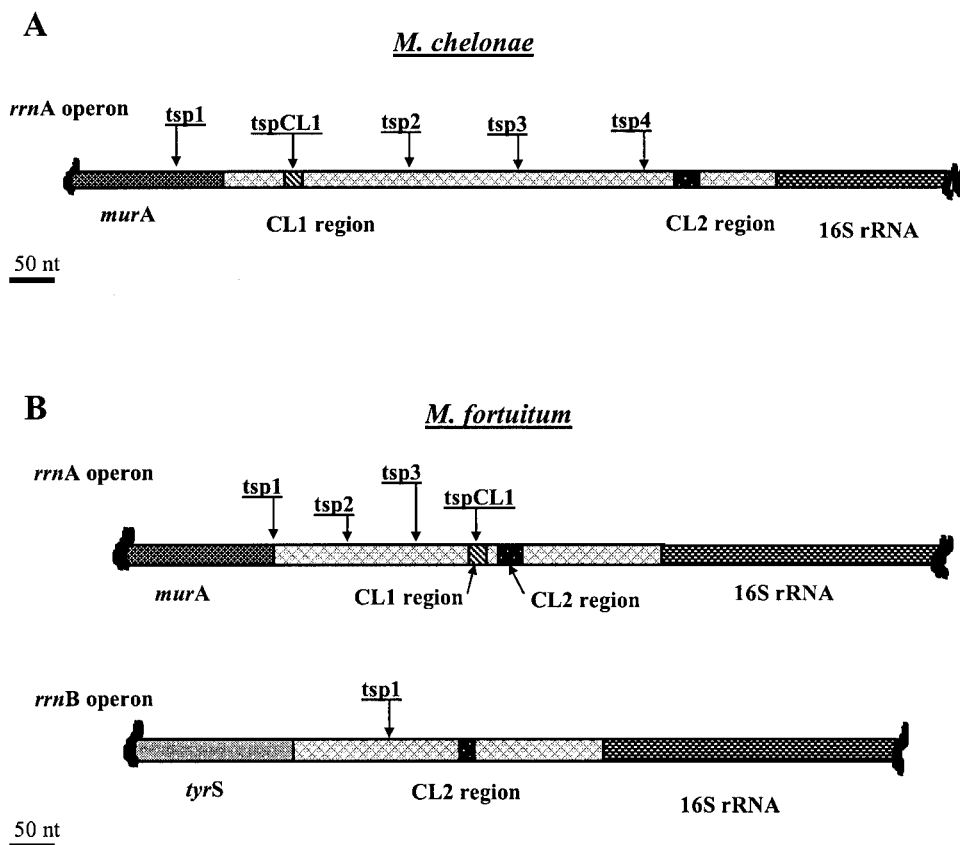


FIG. 1. Schematic representations of the *M. chelonae* and *M. fortuitum* *rrm* operons. Genomic organization of the single and the two ribosomal operons corresponding to *M. chelonae* (A) and *M. fortuitum* (B), respectively. Main sequence features are indicated: 16S rRNA coding region, upstream genes (*murA* in *rrnA*; *tyrS* in *rrnB*), approximate locations of the several promoters, and locations of the conserved leader regions CL1 and CL2. See Gonzalez-y-Merchand et al. (13) for details.

motor driving *rrnB* operon synthesis. During exponential growth, one promoter from each operon contributes equally to the rRNA content. However, in stationary phase, *rrnA* dominates, with two of its three promoters contributing more equally to the total rRNA content (14). These results indicate that the tandem promoters driving the *rrnA* operon are not used equally in either pathogenic (*M. tuberculosis*) or saprophytic (*M. smegmatis*) mycobacteria.

The ability to survive inside macrophages and establish a persistent infection in the host is one property that contributes to the success of *M. tuberculosis* as a pathogen (10, 27). *M. avium* is also able to enter and reside inside macrophages (25). Other rapidly growing saprophytic mycobacteria, such as *M. smegmatis*, are unable to maintain a long-term infection inside macrophages (18). Another fast-growing mycobacterium, *M. fortuitum*, is able to enter and remain inside macrophages until it is killed by activated phagocytic cells (8, 21).

M. chelonae and *M. fortuitum* are fast-growing opportunistic pathogenic mycobacteria. Each species has an *rrm* operon belonging to the *rrnA* family, and *M. fortuitum* has a second *rrm* operon, which belongs to the *rrnB* family. In each species pre-rRNA synthesis is regulated by five promoters, distributed into one or two operons, respectively (13) (Fig. 1). These rapidly growing mycobacteria are frequently involved in human infections (4). They are considered typical fast growers,

but with a higher *rrm* promoter content than other fast growers such as *M. smegmatis*, *M. neoaurum*, and *M. phlei* (13).

Although *M. chelonae* and *M. fortuitum* have different numbers of ribosomal operons, they have an identical number of ribosomal promoters. We have now analyzed their promoter usage in cultures grown in several different media and at different stages of growth. A method was developed for the analysis of the mycobacterial pre-rRNA fraction by real-time PCR. This method was applied to macrophages infected with *M. fortuitum*.

Scrutiny of rRNA promoter sequences allowed identification of nucleotide motifs associated with promoter strength (1, 16). Several *rrm* mycobacterial promoters, but not *rrnA* P1, contain sequence motifs that characterize promoters ranging from medium to high levels of expression. We have found that the *rrnA* P1 promoter was always used at a low and sustained level in both the species studied. Expression from the pre-*rrnA* P1 promoter was used as a standard in a real-time PCR study of pre-rRNA synthesis when *M. fortuitum* was grown in macrophages.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. chelonae* ATCC 35752^T, *M. fortuitum* ATCC 6841^T, and *M. bovis* BCG Pasteur were maintained on Löwenstein-Jensen slants at 4°C for short-term storage and in 40% glycerol at -70°C for

TABLE 1. ODs of cultures at which bacteria were recovered and RNA was isolated

Stage of growth	OD ₆₄₀ ^a for the following medium ^b and species:						
	Lemco		Sauton		Kohn-Harris		Midl-7H9, <i>M. bovis</i> BCG
	<i>M. fortuitum</i>	<i>M. chelonae</i>	<i>M. fortuitum</i>	<i>M. chelonae</i>	<i>M. fortuitum</i>	<i>M. chelonae</i>	
A	0.41	0.5	0.38	0.16	0.13	0.24	ND
B	1.14	1.4	0.74	1.28	0.4	0.47	0.45
C	2.5	2.2	ND	ND	0.64	0.72	ND
D	3.32	3.4	1.4	4.1	1.5	2.8	3.2

^a ND, not determined.

^b Midl-7H9, Middlebrook 7H9 medium plus Tween 80 supplemented with ADC.

long-term storage. Rapidly growing mycobacteria were grown in three different media, which can be differentiated by their nutrient contents: a nutrient-rich medium (Lemco broth containing 0.1% [vol/vol] Tween 80), a nutrient-intermediate medium (Sauton broth [5]), and a nutrient-depleted minimal medium (Kohn-Harris glucose medium [14]). Rapidly growing mycobacteria were incubated in the different media at 30°C (*M. chelonae*) or 37°C (*M. fortuitum*) with shaking at 160 rpm. Growth curves were determined by using initial inocula with an optical density at 640 nm (OD₆₄₀) of 0.005. Experiments corresponding to each medium were performed at least twice.

M. bovis BCG Pasteur was grown in Middlebrook 7H9 medium plus Tween 80 (0.05%) supplemented with albumin-dextrose-catalase (ADC; Difco) at 37°C with shaking at 120 rpm. Glass beads (diameter, 4 mm) were added to culture flasks to avoid clumps.

Macrophage culture and mycobacterial infection. *M. fortuitum* was cultured in Dubos-Tween-albumin (DTA) broth medium at 37°C with shaking at 160 rpm. Bacteria were collected when growing exponentially (OD₆₄₀, 0.5 to 0.8) and resuspended in a cell culture medium (Dulbecco's modified Eagle medium [DMEM]) supplemented with 10% fetal calf serum, without antibiotics added. Aliquots (2×10^7 bacteria/ml) were stored at -70°C. The J774 murine macrophage cell line was grown as monolayers for storage in the same medium (DMEM) supplemented with 10% fetal calf serum, 5% dimethyl sulfoxide, and antibiotics; macrophages were stored in aliquots (2.5×10^9 to 4×10^9 cells/ml) in liquid nitrogen.

Macrophages were collected and distributed in flasks before infection. They were further incubated at 37°C overnight. Fresh warmed medium was added before the infection with mycobacteria. Mycobacterial cultures were thawed at 37°C. Bacterial clumps were taken apart by shaking in Fast Prep (45 s; 6.5 m/s) in the presence of 1-mm-diameter glass beads. The viability of the bacteria was confirmed afterwards by plating on Lemco agar. Infection experiments were carried out by using a proportion of 1:0.8 (macrophages to bacilli) as the multiplicity of infection; antibiotic interference with bacterial growth was avoided by excluding antibiotics from cell monolayers for at least three passes before infection. Moreover, no antibiotics were added to infected macrophage cultures. After 2 h of incubation, extracellular bacteria were eliminated by washes with fresh warmed medium. The efficiency of the phagocytosis was checked by acid-fast staining of the macrophage monolayer. No colonies were obtained from the supernatants except when infected cultures were left overnight.

RNA isolation. Mycobacterial in vitro cultures were collected, and total bacterial RNA was isolated as described previously (12). RNA was isolated and analyzed at appropriate intervals representative of several stages of growth: the early-balanced (A), balanced (B), early-stationary (C), and stationary (D) phases. Table 1 summarizes ODs at which bacteria were recovered in each medium for RNA isolation.

M. fortuitum was obtained from infected macrophages that were lysed with a guanidium isocyanate solution supplemented with Tween 80 at five different times, (1, 3, 4, 5, and 7 h) after infection. Bacterial suspensions were resuspended in a guanidium isocyanate solution without Tween 80, and total RNA from intracellular mycobacteria was purified as described previously (12). Mycobacterial RNA from in vitro cultures or infected macrophages was isolated from two to four experiments.

Analysis of precursor rRNA (pre-*rrn*) by primer extension. The ribosomal promoter usage of *M. fortuitum* and *M. chelonae* cultures in the three different media tested was determined by using a PE procedure. The primer used for that analysis, JY15 (5'-CAC-ACT-ATT-GAG-TTC-TC-3'), has a target site that is part of the CL2 region described previously; this region is present in all mycobacterial *rrn* operons studied thus far (13). This primer was end labeled with [γ -³²P]ATP by T4 polynucleotide kinase, and primer extension was carried out

with avian myeloblastosis virus (AMV) reverse transcriptase (Promega) as described previously (12). PE experiments were performed by using 12 to 25 μ g of RNA per experiment, according to the final amount of RNA isolated. Transcriptional products, corresponding to each *rrn* promoter, were quantified by using an Instant Imager system (Packard-Izasa).

Analysis of precursor rRNA (pre-*rrn*) by quantitative real-time PCR. Quantitative real-time PCR was also used to determine the amounts of pre-*rrn* products isolated from *M. fortuitum* and *M. bovis* BCG. The amplification of target sequences was detected by using SYBR green (Light Cycler; Roche), an intercalating dye that binds only to double-stranded DNA, producing an increase in fluorescence as the amount of the PCR product increases. The specificity of the reaction was checked by analysis of the melting curve of the final amplified product. Mycobacterial RNA isolated was reverse transcribed by using AMV reverse transcriptase and random primer hexamers (Promega). The absence of DNA following DNase treatment was checked, before reverse transcription, by performing conventional PCR using oligonucleotide KK4 as the forward primer and RAC8 as the reverse primer (Table 2). These oligonucleotides have their target sequences at the 16S rRNA coding region, nucleotides 6 to 27 and 340 to 359, respectively, according to the *E. coli* positions. PCR cycling was performed as follows: a denaturation step at 95°C for 5 min; 30 cycles of 95°C for 1 min, 58°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The RNA product was considered suitable for reverse transcription when the control-DNA PCR was negative.

Conditions used to perform quantitative real-time PCR were as follows. PCR Master Mix (1 μ l) was supplemented with 3.5 mM (final concentration) MgCl₂ and 0.5 μ M each primer. Sample cDNA (3 μ l) was added to 7 μ l of Master Mix. The PCR cycling program was as follows: denaturation, 1 cycle of 95°C for 10 min with a transition rate of 20°C/s; amplification, 45 cycles at 95°C for 0 s (transition rate of 20°C/s) and at the annealing temperature (Table 2) for 5 s (*rrnA* P1 from both *M. bovis* BCG and *M. fortuitum*) or 10 s (*rrnA* and *rrnB* from *M. fortuitum*) with a transition rate of 20°C/s; extension at 72°C for 12 s (transition rate of 20°C/s) with a single fluorescence acquisition. Fluorescence corresponding to *sigA* amplifications was acquired at 89°C. Amplification cycles were stopped when the negative tube showed increasing fluorescence. The specificity of the amplification was also tested by determining the melting curve on the amplicon after each experiment was finished. One cycle from 50°C (30 s with a transition rate of 20°C/s) to 95°C (0 s at 0.20°C/s) with continuous fluorescence acquisition was performed to obtain corresponding melting curves of the products.

Oligonucleotides used for *M. fortuitum* and *M. bovis* BCG amplifications by real-time PCR are given in Table 2.

Finally, the *M. fortuitum sigA* gene was also amplified. Oligonucleotides *sigA*-F and *sigA*-R (Table 2), taken from the *M. tuberculosis* gene sequence (nucleotides 872 to 891 and 1116 to 1136, respectively, from the *M. tuberculosis sigA* coding sequence), were used for amplification of the *sigA* gene. Oligonucleotides were selected after the most conserved region of that gene was checked. The conserved region of *sigA* in mycobacteria was selected by performing pileup analysis of the gene's sequence in several mycobacteria, such as *M. tuberculosis*, *M. avium* subsp. *avium*, *M. bovis*, *M. avium* subsp. *paratuberculosis*, *M. leprae*, and *M. smegmatis*. A 263-bp amplicon was amplified. The sequence of the amplicon obtained with *M. fortuitum* DNA as a template demonstrated that a fragment homologous to the *sigA* gene was amplified. We infer that the *M. fortuitum sigA* gene was amplified. The same oligonucleotides were used for real-time PCR amplifications.

Amplifications using real-time PCR were performed at least twice per each set of primers and cDNA sample. Data are displayed as means in the figures.

The amount of chromosomal equivalents (cDNA copies) was calculated by

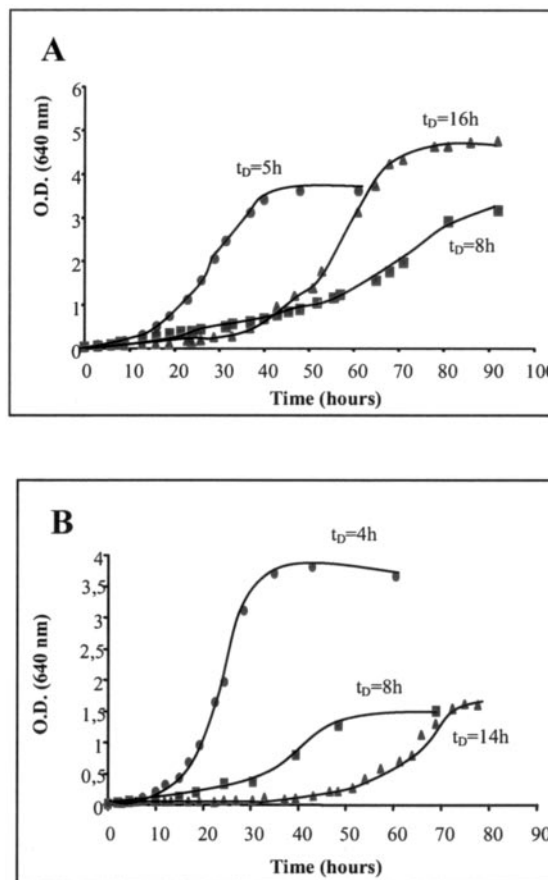


FIG. 2. Growth curves of mycobacteria in different media. Representative growth curves of *M. chelonae* (A) and *M. fortuitum* (B) in the three media used for culture analysis. Circles, Lemco broth; squares, Sauton broth; triangles, Kohn-Harris broth. Doubling times (t_D) for each medium are indicated.

considering the lengths of the chromosomes of *M. bovis* (4.4 Mb) (11) and *M. smegmatis* (7 Mb) (www.tigr.org), which are roughly identical to those of *M. bovis* BCG and *M. fortuitum*, respectively. In accordance with suppliers' recommendations on the accuracy of data, only samples with more than 100 copies were considered for calculations (Roche Diagnostics). In all cases, the negative control was undetectable, because the PCR experiments were stopped before its fluorescence increased.

RESULTS

Growth curves and growth rates. Figure 2A shows representative growth curves of *M. chelonae* cultured in different media with nutrient contents ranging from low (Kohn-Harris minimal medium) through intermediate (Sauton medium) to high (Lemco medium). Figure 2B shows representative growth curves of *M. fortuitum* cultured in the same media. The maximum density of *M. fortuitum* cells was related to the nutritional content of the medium. However, the density of *M. chelonae* rose to similar levels regardless of the nutrient content of the medium. The doubling times of the bacteria in different media were also determined and are given in Fig. 2. As expected, the generation time of each species was longer when it was grown in a medium with a minimal nutrient content, such as Kohn-

TABLE 2. Oligonucleotides used for real-time PCR analysis in this study

Mycobacterium	DNA target	Forward primer ^a	Reverse primer ^a	PCR product (bp)	Annealing temp (°C)
<i>M. fortuitum</i>	<i>rns</i>	KK4 (+6): 5'-GGAGAGATTGGATCCCTGGCTC-3'	RAC8 (+340): 5'-CACTGCTGCCTCCCGTAGG-3'	350	58
	<i>pre-rnaB</i>	FOB10 (-194): 5'-TTTATAGCCCGGGGATTTCT-3'	FOB11 (-112): 5'-AAGAGCGTGGCCCAAAAAACA-3'	101	54
	<i>pre-rna4^b</i>	FORP1 (-181): 5'-CAAAAACAGGGGGCCAAAAGC-3'	eKK4 (+6): 5'-GAGCCAGGATCAAAACTCTCC-3'	200	58
	<i>pre-rna4</i> P1	FOP1 (-411): 5'-CTGCTCGTCAGCCTCGAAAATCG-3'	P1Rv (-358): 5'-ACTTCAAAAGATTAGCGCGG-3'	76	56
	<i>sig4</i>	sig4-F: 5'-CGAAAAAACCATCTCTGGGA-3'	sig4-R: 5'-CTTGTGATCACTCGAACCA-3'	263	58
<i>M. bovis</i> BCG	<i>pre-rna4</i> P1	TBP1 (-231): 5'-ATATCTATGATGACCCGAAC-3'	JY15 (-133): 5'-CACACTATTGAGTTCTC-3'	116	54

^a Numbers in parentheses indicate distances from the 5' ends of 16S rRNA coding regions to the closest binding site of the corresponding oligonucleotide.

^b These primers detect the pool of transcriptional products of *rna4* originating from promoters P1, P2, P3, and PCL1.

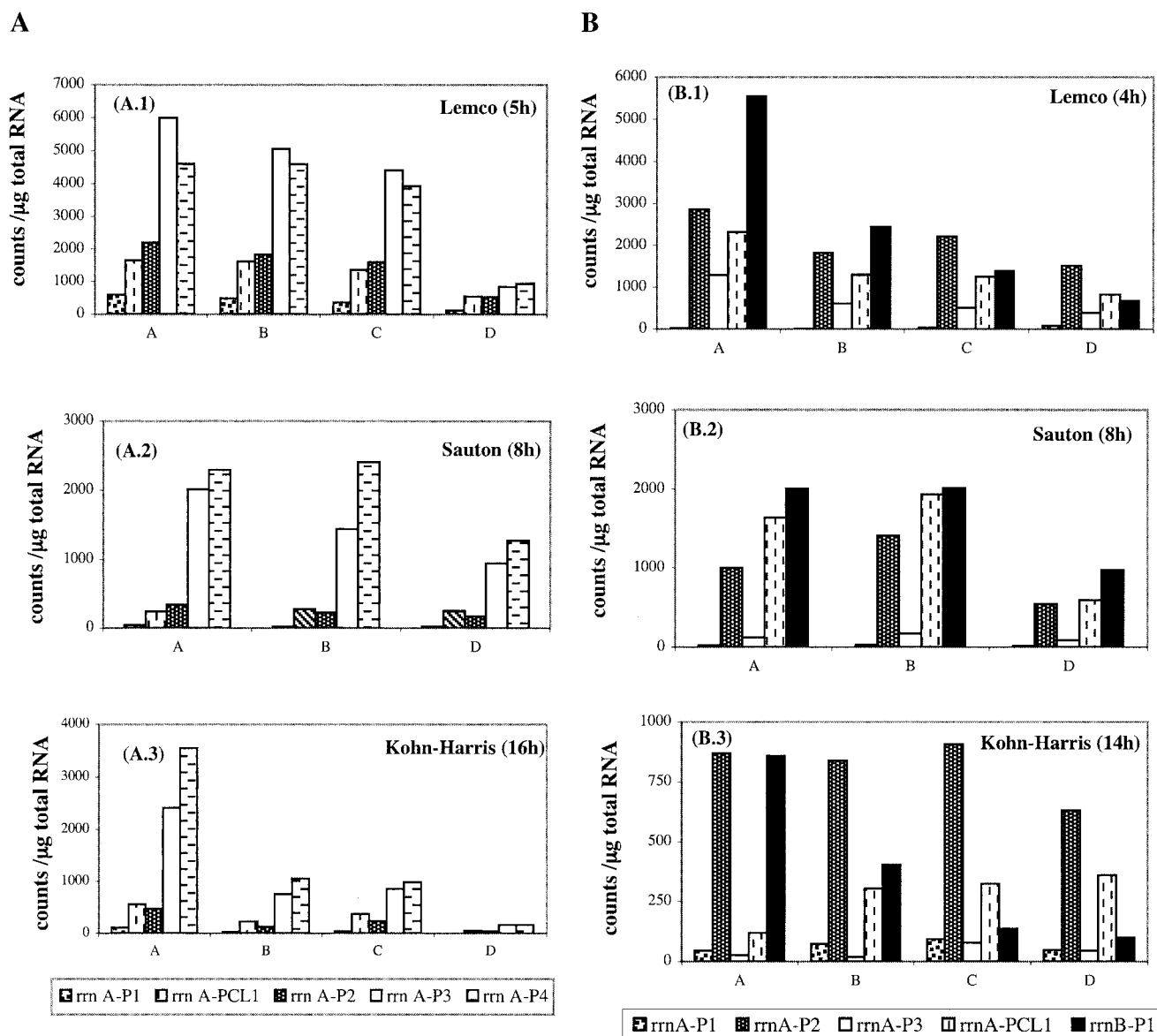


FIG. 3. Promoter usage of *rrn* operons of mycobacteria growing in three different media. Radioactivity of the primer extension products derived from pre-rRNA species of *M. chelonae* (A) and *M. fortuitum* (B). Counts per microgram of total RNA analyzed, corresponding to representative experiments, are given for each five promoters. Amounts of primer extension products were determined in bacterial cultures grown in Lemco medium (A.1 and B.1), Sauton medium (A.2 and B.2), and Kohn-Harris minimal medium (A.3 and B.3), at different stages of growth, as indicated by letters along the x axis (see Materials and Methods). Optical densities corresponding to the different stages of growth are given in Table 1. Doubling times for each culture medium are given in parentheses.

Harris medium. Irrespective of the difference in the number of *rrn* operons per genome, the generation time of each species was found to range from 4 or 5 h to 14 or 16 h according to the growth condition.

Contributions of individual promoters to pre-rRNA synthesis of mycobacteria growing in different media. RNA was isolated at different times of bacterial growth (see Table 1). The relative activities of *rrn* promoters were determined by quantification of the radioactivity levels corresponding to different PE products. Levels of usage for each pre-rRNA promoter product were determined for *M. chelonae* and are shown in Fig. 3A. The *rrnA* P1 promoter seemed to be very poorly used

under all conditions tested, and other promoters were differentially expressed. The proportional contribution of the P1 promoter to pre-rRNA synthesis was in the range of 1 to 4% according to the growth condition. Promoters *rrnA* P3 and P4 were preferentially used irrespective of the nutrient content of the medium or the stage of growth; *rrnA* P3 was usually the more prevalent in bacteria growing in a rich medium, and *rrnA* P4 was more prevalent in the other two media tested.

Results obtained for *M. fortuitum* pre-rRNA synthesis are shown in Fig. 3B and Table 3. In each case, the contribution of *rrnA* to pre-rRNA synthesis exceeded that of *rrnB*. However, under some conditions, the *rrnB* P1 promoter made the largest

TABLE 3. Percentages of the pre-*rrn* fraction contributed by the *rrnA* and *rrnB* operons when *M. fortuitum* was grown under different conditions

Stage of growth and operon	% contributed by the indicated operon in the following growth medium ^a :		
	Lemco	Sauton	Kohn-Harris
A			
<i>rrnA</i>	54 ± 9	59 ± 2	72.5 ± 17
<i>rrnB</i>	46 ± 9.4	41 ± 2	27.5 ± 12
B			
<i>rrnA</i>	57.8 ± 9.4	63.4 ± 3	81.5 ± 13
<i>rrnB</i>	42.2 ± 9.5	36.6 ± 3	18.5 ± 11
C			
<i>rrnA</i>	80.6 ± 14	ND	86.2 ± 4
<i>rrnB</i>	19.4 ± 10	ND	13.8 ± 3.7
D			
<i>rrnA</i>	94.8 ± 3	54.5 ± 4	85.2 ± 0.5
<i>rrnB</i>	5.2 ± 1.3	45.5 ± 4	14.8 ± 0.4

^a The contribution of *rrnA* is the sum of the contributions of each of the four (P1, P2, P3, and PCL1) promoters. Data are mean ± standard deviations from corresponding to two different experiments. ND, not determined. For observed radioactivity levels, see Fig. 3B.

single contribution to pre-rRNA synthesis. Comparisons of the contributions of individual promoters revealed that when *M. fortuitum* was grown in a rich medium, such as Lemco broth, the single promoter of *rrnB* was preferentially used at the exponential phase of growth; as stationary phase approached, the *rrnA* P2 and PCL1 promoters were found to be used more frequently. The contributions of each of these three promoters became more equal when the nutrient content of the medium was lower. For example, during growth in Sauton medium, the *rrnA* P2 and PCL1 promoters were found to contribute approximately 25 and 34%, respectively, to the total transcriptional products (Fig. 3B.2, stages A and B), and the contribution of *rrnB* P1 rose to 46% at stationary phase (Fig. 3B.2, stage D; Table 3). Finally, in a limited-nutrient medium, such as Kohn-Harris medium, the *rrnA* P2 promoter and the single promoter of *rrnB* were preferentially used when bacteria were actively dividing at the early-balanced stage (Fig. 3B.3, stage A). At other stages, *rrnA* P2 was used preferentially vis-à-vis all other promoters. Again, the *rrnA* P1 promoter seems to be little used under all conditions tested; its contribution was in the range of 1 to 10%.

The contributions of *rrnA* and *rrnB* are compared in Table 3. When *M. fortuitum* grew slowly, either in a minimal medium (Kohn-Harris) or at stationary phase (Lemco broth), the *rrnA* operon contributed more to the pool of pre-*rrn* products than *rrnB*. Under other conditions, the contribution of each operon was similar; for example, when *M. fortuitum* was grown in Sauton broth, *rrnA* and *rrnB* accounted for approximately 60 and 40% of pre-rRNA products, respectively, so that the single promoter of the *rrnB* operon was almost as effective as the four *rrnA* promoters taken together.

The concentrations of the radioactive products were found to decrease as the growth rate decreased, reflecting the decrease in the rate of rRNA synthesis. As a general result, the total amount of RNA isolated per OD unit of culture was

found to decrease as the specific growth rate decreased, for example, from the balanced- to the stationary-growth phase, as has been previously reported for other mycobacteria (14).

Suitability of pre-*rrnA* P1 for normalizing the amounts of *M. fortuitum* products in real-time PCR. It was not feasible to identify by quantitative real-time PCR the contributions of the individual promoters of the *rrnA* operon of *M. fortuitum* to pre-rRNA synthesis, because of the difficulty of identifying suitable specific target sequences (see Fig. 1) (13). However, we were able to measure the overall contribution of the four *rrnA* promoters to pre-rRNA synthesis in addition to the contribution of the single promoter of the *rrnB* operon. Experiments were performed with *M. fortuitum* growing in Lemco medium.

The numbers of copies of pre-*rrnA* and pre-*rrnB* per nanogram of total RNA, obtained by using real-time PCR, are shown in Fig. 4A, upper panel (see Table S1 in the supplemental material). For comparison, data for PE analysis are shown (Fig. 4A, lower panel). Similar qualitative patterns of operon usage were obtained by both methods. Each nanogram of total RNA contains 3.4×10^8 copies of the rRNA content (Σ rRNA) of a ribosome (3). The number of copies of pre-rRNA per operon per rRNA content of a ribosome is also shown in Fig. 4A.

Usually transcriptional products are quantified by measuring the actual cDNA level in a sample and using an internal control for normalization. Any reference gene that is expressed in similar amounts under the several conditions tested would be useful for such a purpose. Data obtained by PE experiments (Fig. 3) revealed that, for a particular set of growth conditions, expression corresponding to the *rrnA* P1 promoter was at a low but near-constant level. This promoter differs in two respects from the other *rrn* promoters. First, the -35 box is not a consensus sequence (13), and second, it is G+C rich, in contrast with those of more-active promoters (16). These features are consistent with the low level of expression of this promoter.

The low level of expression of the *rrnA* P1 promoter was found not only in *M. chelonae* and *M. fortuitum* but also in *M. smegmatis* and *M. tuberculosis* (14). These properties of the *rrnA* P1 promoters suggest that these may serve as constitutive promoters suitable for use as standards in real-time PCR. Experiments conducted to test that property were performed with typical mycobacteria carrying 1 (*M. bovis* BCG) or 2 (*M. fortuitum*) *rrn* operons per genome. Analyses were performed with *M. fortuitum* growing rapidly in a rich medium, such as Lemco medium, as well as with bacteria growing at the lowest rate in a nutrient-limited medium, such as Kohn-Harris medium. RNA samples prepared from *M. bovis* BCG cultures growing in Middlebrook 7H9 broth were also tested (see Materials and Methods). Quantification by real-time PCR of cDNA copies corresponding to pre-*rrnA* P1 products is shown in Table 4. The low but sustained level detected for the pre-*rrnA* P1 product indicates its potential suitability as a reference product for comparison to other mRNA mycobacterial transcripts, particularly for comparisons of mRNA products at different stages of bacterial growth in a particular medium.

Accordingly, the pre-*rrnA* P1 product was used to normalize amounts of pre-*rrnA* and pre-*rrnB* when *M. fortuitum* was grown in Lemco medium. The *sigA* gene has been used as a

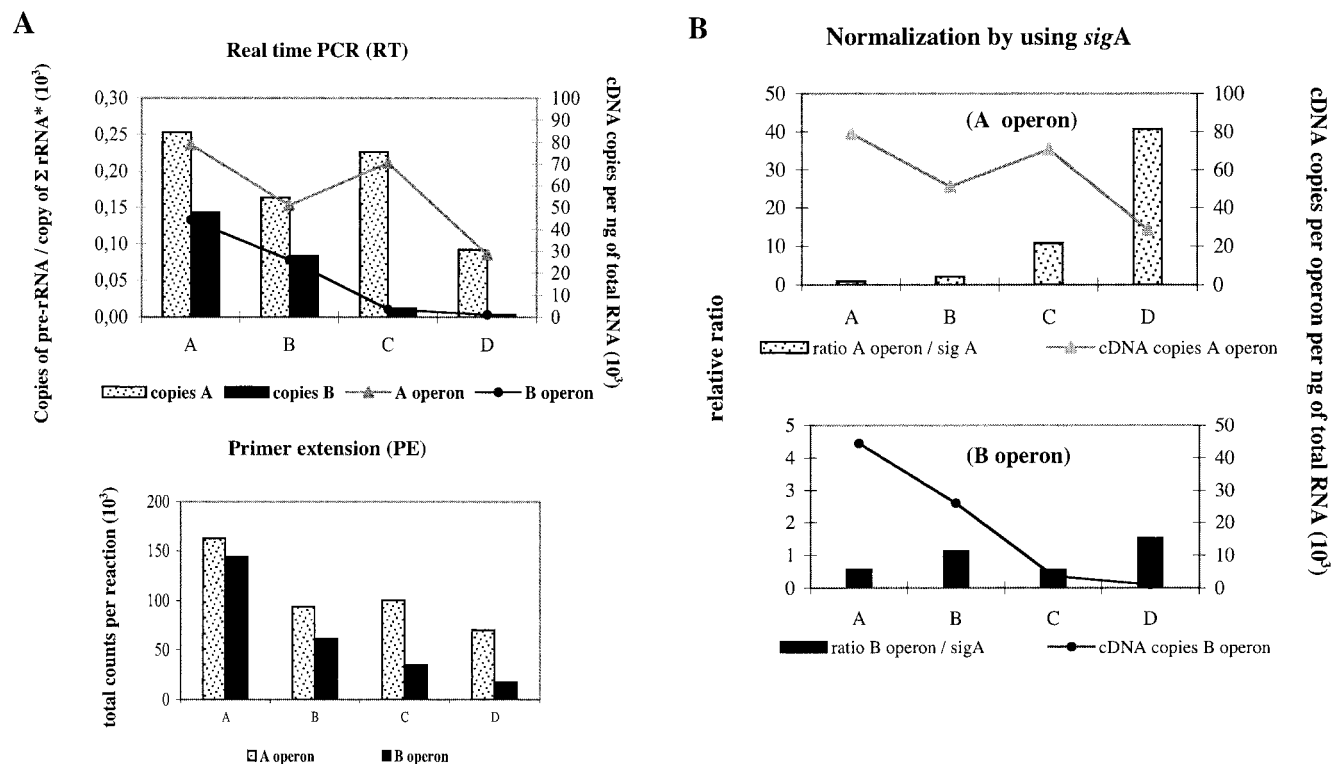


FIG. 4. Evaluation of *rrnA* P1 promoter products as a novel standard for rRNA quantification in *M. fortuitum* grown in Lemco broth. The contribution of the *rrnA* operon to pre-rRNA synthesis is the sum of the contributions of all four (P1, P2, P3, and PCL1) promoters. (A) Comparison of results obtained for the analysis of the pre-rRNA fraction by real-time PCR and the primer extension method. (Upper panel) Bars (left axis), theoretical number of copies (in thousands) of pre-rRNA corresponding to each *rrn* operon per copy of rRNA (as calculated from nanograms of total RNA) (3). Lines (right axis), amounts of product as determined by real-time PCR, expressed as thousands of cDNA copies per nanogram of total RNA (with no reference or normalizing gene used). *, Σ rRNA represents the rRNA content of a ribosome, that is, 1 copy of 16S rRNA, 1 copy of 23S rRNA, and 1 copy of 5S rRNA. The number of copies of Σ rRNA per nanogram of total RNA was calculated on the basis of the assumption that each ribosome contains 4,566 nucleotides with an average mass of 324 Da (3). (Lower panel) Amounts of product are expressed as radioactivity (total counts [in thousands] per reaction). (B) Contributions of *rrnA* and *rrnB* to pre-rRNA synthesis, measured by real-time PCR, normalized to products of *sigA*. Bars (left axis) show data as the *rrnA/sigA* (upper panel) and *rrnB/sigA* (lower panel) ratios. Lines (right axis) show the level of expression (in thousands of cDNA copies per nanogram of total RNA) of the appropriate operon (from panel A, top graph), reproduced here for comparative purposes. (C) Contributions of *rrnA* and *rrnB* to pre-rRNA synthesis, measured by real-time PCR, normalized to products of the P1 promoter of *rrnA*. Bars (left axis) show data as the *rrnA/rrnA* P1 (upper panel) and *rrnB/rrnA* P1 (lower panel) ratios. Lines (right axis) show the level of expression (in thousands of cDNA copies per nanogram of total RNA) of the appropriate operon (from panel A, top graph), reproduced here for comparative purposes. Due to the differences in the amounts of products obtained, different scales had to be used for the two operons in panels B and C.

suitable housekeeping sigma factor for *M. tuberculosis* (24) and has been proposed as a good candidate for such an internal control (19) in exponentially growing cells. However, *sigA* expression was found to decrease threefold upon entry into stationary phase (19). The suitability of *sigA* for quantifying tran-

scripts of *rrn* operons of *M. fortuitum* was tested, and comparison was made with the use of pre-*rrnA* P1 as a reference product. Quantification of results standardized with *sigA* led to values for the transcripts of the *rrnA* and *rrnB* operons (Fig. 4B) that corresponded poorly with the previously re-

TABLE 4. Levels of pre-*rrnA* P1 during mycobacterial growth^a

Stage of growth	Level of pre- <i>rrnA</i> P1 in the following growth medium ^b :				
	Lemco		Kohn-Harris		Midl-7H9, real-time PCR
	Real-time PCR ^c	PE ^d	Real-time PCR	PE	
<i>M. fortuitum</i>					
A	5,496 (27)	5.6	17,661 (13)	5.5	ND
B	4,772 (30)	7.1	25,774 (32)	5.0	ND
C	4,162 (25)	10.6	34,655 (15)	10.1	ND
D	7,333 (19)	13.4	18,379 (28)	6.2	ND
<i>M. bovis</i> BCG					
B	ND	ND	ND	ND	23,460 (29)
D	ND	ND	ND	ND	19,595 (14)

^a Quantification of pre-*rrnA* P1 by real-time PCR during mycobacterial growth and comparison with results obtained by using PE.

^b Midl-7H9, Middle brook 7H9 medium plus Tween 80 supplemented with ADC. ND, not determined.

^c Real-time PCR data are expressed as the number of pre-*rrnA* P1 cDNA copies per nanogram of total RNA; according to suppliers' recommendations on the accuracy of data, only experimental data with more than 100 copies were considered. Each data point is the mean (with the coefficient of variation, expressed as a percentage, in parentheses) from four to six determinations. Statistical significances of data were determined by applying one-way analysis of variance. No significant differences were found when data for the same medium at different stages of growth were compared ($P = 0.43$ for Lemco medium; $P = 0.58$ for Kohn-Harris medium; $P = 0.77$ for Middlebrook 7H9 medium).

^d PE data are expressed as counts per microgram of total RNA, and they correspond to a single representative experiment. Coefficients of variation are 32% for Lemco medium and 34% for Kohn-Harris medium.

ported primer extension and real-time PCR data (Fig. 4A). In contrast, normalization using *rrnA* P1 as a reference product (Fig. 4C) agreed much more closely with those data. The increasing levels of *rrnA* products displayed during stationary phase when *sigA* is used as an internal control could be explained by the decrease in the level of the *sigA* expression relative to that in exponential phase (19).

Mycobacterial pre-rRNA synthesis in mouse macrophages infected with *M. fortuitum*. Murine macrophages (cell line J774) were infected with *M. fortuitum* (0.8 mycobacterium/cell, on average). The number of mycobacteria per cell appeared to increase (up to 2 mycobacteria/cell, on average) during the first 7 h, as judged by optical microscopy. Samples of cells were taken at a series of time intervals; total RNA was isolated, and cDNA was prepared (see Materials and Methods). The primer extension method could not be used to analyze the pre-rRNA fraction of *M. fortuitum* because of the low concentration of mycobacterial RNA relative to mouse RNA. For this reason the levels of *M. fortuitum* pre-rRNA were measured by real-time PCR. An appropriate reference product, pre-rRNA transcripts of the P1 promoter of *rrnA*, was used. In this way the relative contributions of *rrnA* and *rrnB* to pre-rRNA synthesis were estimated. After the first hour of infection, the contribution of *rrnA* to pre-rRNA synthesis exceeded that of *rrnB*. After 3 h of infection, *rrnB* became the major contributor (75% or more) to pre-rRNA synthesis (Fig. 5) (see Table S2 in the supplemental material). This situation was not observed when pre-rRNA fractions of laboratory-grown cultures of *M. fortuitum* were analyzed. Further investigations are needed to confirm and explain this new pattern of pre-rRNA synthesis.

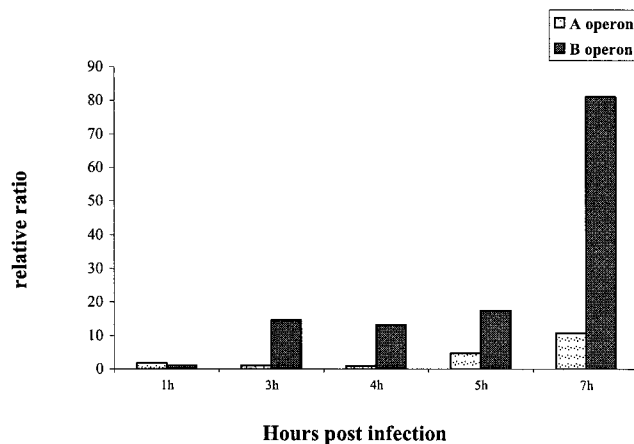


FIG. 5. Synthesis of rRNA in *M. fortuitum* infecting macrophages. The *rrn* transcriptional products in *M. fortuitum* infecting macrophages were quantified by real-time PCR. RNA from intracellular bacteria was isolated and quantified after several hours postinfection. Relative ratios (*rrnA/rrnA* P1 and *rrnB/rrnA* P1) obtained by using pre-*rrnA* P1 for normalization are shown. The transcriptional products of the *rrnA* operon are the sum of the contributions of its four promoters (P1, P2, P3, and PCL1).

DISCUSSION

E. coli, which is often used as a model bacterium, has 7 *rrn* operons per genome, and each is controlled by two tandem promoters, P1 and P2; the promoter regions share a high degree of sequence similarity and, at least according to preliminary studies, all the operons may be regulated in similar ways (6).

In contrast, mycobacteria have either 1 or 2 *rrn* operons per genome, *rrnA* and/or *rrnB*, which are regulated in different ways. All the mycobacteria studied to date have an *rrnA* operon regulated by two (e.g., *M. tuberculosis*) to five (e.g., *M. chelonae*) tandem promoters. Some mycobacteria, for example, *M. fortuitum*, have a second operon, *rrnB*, regulated by a single promoter (20).

Function of multiple tandem promoters. Two promoters, P1 and PCL1, are common to all *rrnA* operons investigated thus far. The P1 promoter is located within the coding region and/or near the 3' end of the upstream (*murA*) gene (13). The P1 promoter differs from all other *rrnA* promoters in that the -35 box is not a consensus sequence, and so an additional factor(s) may be needed to initiate transcription. The PCL1 promoter is characterized by a conserved leader sequence downstream from its -10 box. The *rrnA* operon of *M. tuberculosis* is regulated by the two promoters P1 and PCL1 only. In contrast, the *rrnA* operon of *M. chelonae* is regulated by P1, PCL1, and three additional promoters, P2, P3, and P4. Four (P2, P3, P4, and PCL1) of the five promoters of the *rrnA* operon of *M. chelonae* have -10 boxes "extended" by the TGN motif at the 5' end. The presence of the TGN extension is thought to enhance promoter strength (1, 17, 23). Extended -10 boxes are present in the promoters of *rrn* operons of *M. chelonae* and *M. abscessus* but not in other mycobacterial *rrn* operons (13). That motif could be acquired by these mycobacteria during evolutionary adaptation to rapid growth, thus compensating for the disadvantage of having a single ribosomal operon. Two of the five

promoters of *M. chelonae*, P3 and P4, were found to contribute most to pre-rRNA synthesis irrespective of growth conditions or the stage of growth (Fig. 3A). There are insufficient data to explain why promoters P3 and P4 contribute so strongly to pre-rRNA synthesis. The interactions among the five promoters of *M. chelonae* are likely to be more difficult to unravel than the interactions between the two tandem promoters of *E. coli* (28).

In contrast with *M. chelonae*, *M. fortuitum* has 2 *rm* operons per genome; the *rmA* operon is regulated by four promoters, P1, P2, P3, and PCL1, and *rmB* has a single promoter (20). The P1 promoter of *rmA* was utilized at a low level, as in the case of *M. chelonae*, so that P2, P3, and PCL1 were the main regulators of *rmA* expression. When *M. fortuitum* was grown in the laboratory, the contributions of *rmA* and *rmB* to pre-rRNA synthesis depended on the growth rate. In a rich medium, when growth was balanced, the two operons contributed more or less equally to pre-rRNA synthesis, whereas in less nutritionally rich media or at a low rate of growth, the *rmA* operon was the principal contributor. At low rates of initiation of pre-rRNA synthesis, the four promoters of *rmA* would be expected to increase the probability of binding RNA polymerase over that of *rmB*. At high rates of initiation of pre-rRNA synthesis, occlusion effects between tandem promoters are expected to diminish the advantage for the *rmA* operon of possessing multiple promoters (14); in contrast, the efficiency of usage of the single *rmB* promoter would not be affected (Table 3 and Fig. 3B).

Transcripts regulated by the P1 promoter of *rmA* provide a standard for quantitative real-time PCR. It was not feasible to study the pre-rRNA fraction of *M. fortuitum* grown in macrophages by means of the primer extension method. Investigation by real-time PCR was appropriate provided that a normalizing procedure was identified; this step was needed to relate the number of mycobacterial pre-rRNA transcripts to the small amount of *M. fortuitum* cDNA present in cDNA copies from the RNA fraction isolated from infected mouse macrophages. Transcripts controlled by the P1 promoter of the *rmA* operon were used as a suitable reference species for a particular set of growth conditions, because they represent a small but largely constant amount of the pre-rRNA fraction (Table 4).

Absolute comparisons between Lemco and Kohn-Harris media are difficult to make without establishing a frame of reference that is common to both, because of intrinsic differences between the two media. However, the data in Table 4 suggest that a factor of approximately 5 could be applied to reconcile the two sets of data.

When comparison was possible, the results obtained by real-time PCR were in general agreement with the results obtained by the primer extension method (see Fig. 4A). It was estimated that 1 ng of the RNA fraction of *M. fortuitum* comprises 3.4×10^8 copies of the RNA complement of a ribosome (3). The results (see Table 4) of real-time PCR suggest that the number of *rmA* P1-directed transcripts is at least $\sim 5,000$ or $\sim 25,000$ copies per ng of total RNA or per 3.4×10^8 ribosomes and differs depending on the growth medium and conditions. Thus, the number of copies of pre-rRNA directed by the *rmA* P1 promoter per nanogram of total RNA suggests that the *rmA* P1 promoter product is a suitable standard for quantification

of many mycobacterial mRNA species. Thus, *rmA* P1 is a useful low-abundance reference product, present as a single copy per genome in all mycobacteria analyzed thus far. It is particularly suitable for normalizing the levels of gene expression at different stages of mycobacterial growth (early-balanced to stationary phase) in a particular growth medium, such as Lemco broth or Kohn-Harris medium.

Analysis of the pre-rRNA fraction of *M. fortuitum* infecting macrophages. The composition of the pre-rRNA fraction of *M. fortuitum* infecting macrophages was determined by real-time PCR using the products of the P1 promoter of *rmA* as the reference. After the first hour of infection, the contribution of *rmA* to pre-rRNA synthesis exceeded that of *rmB*, in agreement with the results obtained for laboratory-grown *M. fortuitum*. After 3 h of infection, the *rmB* operon provided the majority (75% or more) of the pre-rRNA transcripts (Fig. 5), showing that, compared with laboratory culture, growth in macrophages alters the balance between the contributions of the *rmA* and *rmB* operons.

It is inferred from the increase in the number of mycobacteria per cell observed by microscopy that the doubling time of *M. fortuitum* was approximately 7 h during the infection, comparable to the growth rate observed for laboratory culture in a rich medium. Finally, we have developed a method for analyzing the pre-rRNA fraction of *M. fortuitum* infecting macrophages. Compared to those for laboratory growth, significant differences in the contributions of the *rmA* and *rmB* operons were observed; this pattern merits further investigation. There are no previous studies analyzing rRNA synthesis in mycobacteria infecting macrophages; such studies are of interest because they will help to increase our knowledge of how mycobacteria survive inside phagocytic cells.

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