The pathogen *Mycobacterium marinum*, a faster growing close relative of *Mycobacterium tuberculosis*, has a single rRNA operon per genome

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Abstract

Although *Mycobacterium marinum* and *Mycobacterium tuberculosis* are very closely related they differ significantly in their growth rates. The Type strain of *M. marinum* and one clinical isolate were investigated and, like *M. tuberculosis*, were found to have a single rRNA (*rrn*) operon per genome located downstream from *murA* gene and controlled by two promoters. No sequence differences were found that account for the difference in the growth rates of the two species. We infer that *M. tuberculosis* has the capacity to synthesize rRNA much faster than it actually does; and propose that the high number of insertion sequences in this species attenuate growth rate to lower values.

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1. Introduction

Mycobacteria are non-motile, rod-shaped bacteria that are aerobic, Gram-positive and acid-fast [1]. The genus comprises approx. 75 species that are closely related as judged by the similarities in their 16S rRNA sequences [2]. Usually, mycobacteria are classified as either fast growing or slow growing according to whether colonies are seen on a solid medium within five days (fast growers) or longer (slow growers). Slow growers include the human pathogens *Mycobacterium leprae* and *Mycobacterium tuberculosis* which have generation times of 12 days [3] and approaching one day [4], respectively. *Mycobacterium marinum* causes cutaneous granulomas, papules or nodules in man and is pathogenic for mammals, reptiles, amphibians and fish [1,5,6]. *M. marinum* is very closely related to the slow growing pathogen, *Mycobacterium ulcerans*, and both are thought to be the closest relatives of *M. tuberculosis* as revealed by 16S rRNA sequences (99.3% sequence similarity, 11 nucleotide differences [7]), DNA/DNA hybridization and fatty acid profile analysis [8]. *M. tuberculosis* and *M. marinum* share many features of pathophysiology, including the capacity to survive and proliferate within host cells [9–11]. For these reasons *M. marinum* has been considered as an appropriate model for studying the pathogenicity of *M. tuberculosis* [12,13].

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In vivo *M. marinum* was found to have a generation time of 24 h at 30 °C [5,6] comparable with that of *M. tuberculosis*. However, their generation times are strikingly different when they are grown in the laboratory. Whereas the generation time of *M. tuberculosis* grown in liquid cultures at 37 °C approaches 24 h, *M. marinum*, which grows optimally at 32 °C, has a generation time of 4 h [5,6]. This difference in the growth rates has yet to be explained.

During bacilli replication, approximately half the energy expended is devoted to protein biosynthesis [14]; proteins comprise almost half of a bacterial cell’s dry mass. In turn, the synthesis of proteins requires the synthesis of new ribosomes. For instance, the rate of specific protein synthesis is directly proportional to the third power of the ribosome concentration [15]. It is thought that the rate determining step in ribosome synthesis is the rate of rRNA synthesis [16].

One mechanism whereby growth rates and ribosome synthesis rates may be linked is through gene dosage with fast growing mycobacteria having two rRNA (rrn) operons per genome and slow growers having only one [17,18]. Synthesis of rRNA is also known to be regulated at the level of gene expression through the characteristic features of the promoters governing precursor-rRNA (pre-rRNA) synthesis.

The tubercle bacillus is known to have a single rrn operon per genome [17]. The near fivefold difference in the growth rates of *M. marinum* and *M. tuberculosis* prompted us to identify the number of rrn operons that are present in the genome of *M. marinum*, the transcription start points and the promoters engaged in pre-rRNA synthesis.

### 2. Materials and methods

Materials were obtained commercially.

#### 2.1. Mycobacterial strains

The type strain *M. marinum* ATCC 927T and one clinical isolate given by Dr. Ruth Parra of the National Medical Center (Mexico City) were maintained on Lowenstein–Jensen slopes at 4 °C and, when required, were grown at 32 °C in Dubos broth containing 0.1% (w/v) Tween 80 and ADC [0.5% (w/v) bovine serum albumin/0.2% (w/v) dextrose/0.004% (w/v) catalase]. Cells were harvested in early exponential phase for RNA isolation and in early stationary phase for DNA isolation.

#### 2.2. Isolation of genomic DNA

Genomic DNA was isolated from mycobacterial cells by a guanidinium chloride method [19]. Briefly, cells were collected by centrifugation at 13,000g during 20 min and resuspended in lysis buffer [6 M guanidinium chloride, 10 mM EDTA, 0.1% (v/v) Tween 80, 1 mM 2-mercaptoethanol]. The cell suspension was transferred from an ethanol/CO₂ freezing mixture to a water bath at 65 °C for 10 min and then cooled in ice bath for 5 min. This suspension was extracted twice with 2 vol of chloroform/isoamyl alcohol (24:1 v/v). DNA was precipitated with 2.5 vol of cold ethanol and left at −20 °C overnight. The DNA precipitate was recovered by centrifugation and dissolved in 1X TE buffer (10 mM TRIS, 1 mM EDTA) and stored at −20 °C.

#### 2.3. Restriction enzyme digestion of DNA and Southern blot transfer

Samples of DNA isolated from the strains of *M. marinum* and a sample of DNA isolated from *M. tuberculosis* H37Rv (given by our colleague S. Rivera-Gutierrez) were digested with either 10 units of *Pst*I or 10 units of *BamHI* per microgram of DNA. The fragments were separated by electrophoresis through 1% (w/v) agarose gel and transferred to a nylon membrane by the Southern procedure.

#### 2.4. Preparation of radioactive probes

##### 2.4.1. Amplification of probe sequences

The conditions used for amplification of DNA sequences by the polymerase chain reaction were described previously [20]. Two probes were used. One probe was specific for 16S rRNA sequences amplified by the primers RAC103 (5'-GTC GAA 3') and RAC6 (5'-GGT GGA CTA CCA GGT TAA CAC ATG CAA -3') which amplified a fragment of *M. marinum* DNA corresponding to positions 47–799 of the *M. tuberculosis* 16S rRNA gene [21] spanning the *Pst*I site at positions 650–655. The other probe amplified *M. marinum* DNA sequences extending from the 3'-end of the 16S rRNA, internal transcribed spacer 1, and the 5'-end of the 23S RNA gene. The primer combination 5RI (5’ GGA GTC GTA ACA AGG TAG CCG 3’) and 3RI (5’ TGC CAA GGC ATC CAC C 3’) was used.

##### 2.4.2. Radiolabelling of probe sequences

Each amplicon was labelled as specified in the manual of the Rad Prime Labeling System (Invitrogen); DNA was denatured and repaired using Random Primers, with the Klenow fragment in the presence of [α³²-P]dCTP (obtained from Amersham International).

#### 2.5. Hybridization after Southern transfer

After Southern transfer, the DNA fragments were attached to a membrane by UV irradiation [22]. Membranes were pre-treated at 65 °C for 1 h in 5× Denhardt’s...
solution and salmon sperm (80 μg/ml) DNA; and then hybridized with the radioactive probe overnight at 65 °C in 5× Denhardt’s solution. After hybridization the membranes were washed once in 0.5 × SSC/0.1% SDS for 20 min at 65 °C; and twice in 0.1 × SSC/0.1% SDS for 5 min at room temperature. The membranes were covered with “Clean Pack” and exposed to a photographic film together with an intensification screen at −70 °C.

2.6. Amplification and sequencing of the region between the murA and 16S rRNA genes of M. marinum

The upstream region of the rrnA operons, comprising 228 nt of murA, the promoter regions and 360 nt from the 5′-end of each of the 16S rRNA coding regions were amplified using primer RAC1 (5′-TCG ATG ATC ACC GAG AAC GTG TTC-3′) in combination with RAC8 (5′-CAC TGG TGC CTC CCG TAG G-3′). The targets for primer RAC1 correspond to the sequences within the murA gene; namely, positions −298 to −275, as shown in Fig. 2. The target for RAC8 corresponds to positions 339 to 357 of the 16S rRNA coding region of M. tuberculosis [21].

Sequences of PCR products were determined both manually and automatically by the dideoxy-chain termination procedure using the commercial kit “Sequenase PCR product sequencing kit” (USB) with appropriate internal primers [23].

2.7. Isolation of RNA

Cells were collected from early exponential phase cultures and resuspended in 1 ml of lysis buffer and total RNA was isolated as described previously [24]. The RNA was redissolved in an appropriate volume of morpholinepropanesulfonic acid (MOPS) buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA; pH 8.3). The integrity of RNA was checked by electrophoresis through formaldehyde gels.

2.8. Primer extension

The oligonucleotide primer JY15 (5′-CAC ACT ATT GAG TTC TC-3′) has a target sequence present in all the mycobacterial rrn operons so far studied (see Fig. 3). This primer was end-labelled with [γ32P]ATP by means of T4 polynucleotide kinase and the primer extension was carried out using the AMV Reverse Transcriptase primer-extension system as described previously [24]. The extension products were separated in an 8% (w/v) polyacrilamide–urea gel and visualized by autoradiography.

2.9. Alignment of sequences

Computer-aided analysis of the alignment of nucleotide sequences of murA and promoter regions was achieved by means of the BLAST program [25].

3. Results and discussion

The Type strain of M. marinum and the clinical isolate were each shown to possess a single rrn operon per genome by examining the patterns of hybridization of restriction endonuclease digests (PstI and BamHI) of DNA to a radio labelled probe.

Fig. 1. Southern-blot transfer of restriction enzyme digests and hybridization reveal that M. marinum has one rrn operon per genome. (a) Hybridization with a 32P-labelled 16S rDNA fragment amplified by using primers RAC6 and RAC103. The probe spans the PstI site from 650 to 655 bp downstream from the 5′end of the 16S rRNA gene. 1, Unrestricted DNA isolated from M. marinum Type strain; 2, PstI digest of M. marinum Type strain DNA; 3, PstI digest of M. tuberculosis H37Rv DNA. (b) Hybridization with a 32P-labelled PCR fragment amplified using primers R15 and R13 spanning internal transcribed spacer 1. 4, PstI digest of M. marinum Type strain DNA; 5, BamHI digest of M. marinum Type strain DNA. (c) Restriction fragment map of the rrn operon of M. marinum Type strain derived from the data of (a) and (b). The DNA within the region enclosed by the box was amplified by PCR and its nucleotide sequence was established.
Two probes hybridizing to different regions of an rrn operon were used. One probe [18], which hybridized to 16S rRNA sequences alone, revealed two target sequences per operon with PstI digests because the probe sequences spanned the PstI site. The second probe identified sequences extending from the 3'-end of the 16S rRNA gene to the 5'-end of the 23S rRNA gene. Representative results are shown in Fig. 1 for the Type strain. Identical results were obtained for both the Type strain and the clinical isolate. For comparison, PstI digests of M. tuberculosis H37Rv were also analyzed (Fig. 1(a)).

Hybridization of PstI digests with the first probe identified two target sequences in samples of M. marinum and M. tuberculosis DNA. Thus, M. marinum, like M. tuberculosis, possesses a single rrn operon per genome.

The results obtained by hybridizing BamHI fragments (Fig. 1(b)) with the second probe confirm that M. marinum has a single rrn operon per genome and also allow the construction of the map of restriction enzymes fragments shown in Fig. 1(c).

All mycobacteria examined to date possess one rrn operon located downstream from the murA gene [26].

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Fig. 2. Sequences of the 3'-region of murA, the intergenic region and the 5'-regions of 16S rRNA of M. marinum (see box in Fig. 1(c)) and comparison with M. tuberculosis. M. ma, M. marinum; M. tu, M. tuberculosis. =, identical nucleotide; –, deletion. Nucleotides are numbered, both upstream (−) and downstream (+) from the transcription start points of the P1 promoters (see Fig. 3). Sequences of murA genes are shown as codons (NCBI GenBank Accession No. AY532648). Termination codons are underlined. The intergenic regions are shown within the box. Promoter elements (−35 boxes, −10 boxes) and transcription start points are indicated by bold upper case letters; the direction of transcription is shown by the superscript bent arrow. The highly conserved sequence motifs CL1 and CL2 are shown in bold lower case letters. The 5'-regions of 16S rRNA are indicated by lower case italics (NCBI GenBank Accession No. AY532649; this sequence and that cited in http://www.sanger.ac.uk/cgi-bin/blast/submitblast/m_marinum are identical).
Sequences near the 3'-end of the murA gene, the intergenic region and the region near the 5'-end of the 16S rRNA gene (the boxed region in Fig. 1(c)) were shown to be readily amplified by PCR [23]. The amplicon (see Fig. 2) is conveniently divided into four regions [27]. First, the murA coding region; second, the hypervariable multiple promoter region extending from the murA stop codon to the start point of the PCL1 promoter; third, the core-leader region which extends from the start point of the PCL1 promoter to the 5'-end of the 16S rRNA gene; and fourth, the sequences (including the V2 variable region) near to the 5'-end of 16S rRNA.

Comparisons of sequences of M. tuberculosis and M. marinum are summarized in Table 1. Amino acid sequences near to the 5'-end of the murA gene are conserved (1 amino acid difference) but synonymous codons were found. Both mycobacteria have two promoters with homologous −10 and −35 boxes. The CL1 sequence motif which is a feature of CL1 promoters is common in both species. The core-leader region has at least two functions and the CL2 motif, which is a signature motif for the genus Mycobacterium, plays a major role in both; it is important to transcriptional control because it is implicated in antitermination and, in precursor rRNA, it forms a pan handle by interacting with sequences within ITS-1 downstream from the 3'-end of 16S rRNA. Both species conform to the common pattern of precursor 16S rRNA described by Ji et al. [20] (result not shown).

Few differences are found in the 16S rRNA coding regions of the two species (99.4% similarities). Although there are numerous sequence differences upstream from the 5'-ends of the 16S rRNA genes they are thought to occur in regions where sequence differences are tolerated without affecting rRNA transcription or ribosome function.

Primer extension analysis of the RNA fraction of both the Type strain and clinical isolate (result not shown) revealed two transcription start points corresponding to the P1 and PCL1 promoters present in all mycobacterial rRNA operons (see Fig. 3).

Table 1
Comparison of sequence differences, including insertions and deletions, found between M. tuberculosis and M. marinum

<table>
<thead>
<tr>
<th>Region</th>
<th>M. tuberculosis</th>
<th>M. marinum</th>
</tr>
</thead>
<tbody>
<tr>
<td>MURA</td>
<td>0/75</td>
<td>1/74</td>
</tr>
<tr>
<td>3'-end of murA</td>
<td>0/225</td>
<td>51/222</td>
</tr>
<tr>
<td>HMPRb</td>
<td>0/77</td>
<td>38/90</td>
</tr>
<tr>
<td>Core leader region</td>
<td>0/177</td>
<td>91/196</td>
</tr>
<tr>
<td>5'-end 16S rRNA</td>
<td>0/305</td>
<td>6/305</td>
</tr>
<tr>
<td>16S rRNA gene</td>
<td>100% similarity</td>
<td>99.3% similarity</td>
</tr>
</tbody>
</table>

*a* The fractions 0/75, etc. denote the number of sequence differences divided by the length of the sequence in the Mycobacterium specified; the M. tuberculosis sequence is used for reference.

*b* HMPR, hypervariable multiple promoter region [27].

*c* Ref. [8].

We infer that since the rRNA operons of both species have very similar promoter elements, each has the same (or at least a very similar) capacity for the synthesis of rRNA. In other words, the rRNA operon of M. tuberculosis appears to have the potential to synthesise pre-rRNA at a rate that would support the generation time (t<sub>0</sub>) of 4 h found for M. marinum.

One possible explanation for the four to fivefold difference in the growth rates of M. marinum and M. tuberculosis is that either the tubercle bacillus has defective genes or has lost genes that support faster growth, as proposed for the very slow growth of M. leprae and for the inability to grow this organism in vitro [28]. The feasibility of this notion was tested by comparing data for the genome sizes of selected mycobacteria (see Table 2); the table also includes the numbers of rRNA operons per genome, the numbers of promoters per cell and generation times (usually in liquid shake cultures).

The comparative data support several conclusions. First, the M. marinum genome (6.02 \times 10^{6} base-pairs) is significantly larger than the M. tuberculosis genome (4.41 \times 10^{6} base pairs). Second, in general the faster growing species have the larger genomes. However, there are several anomalies. First both Mycobacterium chelonae and M. tuberculosis have genomes of approximately 4.4 \times 10^{6} base-pairs but they differ fourfold in their growth rates. Secondly Mycobacterium simiae grows very slowly although its genome size approaches
rate of growth, are in accord with a theoretical analysis of operons per genome have in addition to the number of the rrn operon. These observations, which show that factors other than crp a null mutation of M. tuberculosis, for instance the production of a component for cell wall synthesis, thus rendering the synthesis of that component the rate limiting step during cell proliferation. Such a mutation would not affect the cell's potential for rRNA synthesis. For example, M. tuberculosis carrying a null mutation of the cyclic AMP receptor protein (CRP) gene was found to grow much more slowly than the parental strain [R. S. Buxton, National Institute for Medical Research, Mill Hill, London, UK, unpublished work]. Likewise, a mutant of M. smegmatis

that of M. marinum. Third, although each possesses a single rrn operon per genome both M. marinum and M. chelonea can grow at rates approaching those of typical fast growers, possessing two rrn operons per genome. Fourth, deletion of either one of the two rrn operons of Mycobacterium smegmatis has no effect on growth rate [29]. No correlation was found between either genome sizes, the numbers of rrn operons per genome, or the numbers of promoters per cell and generation times. These observations, which show that factors other than the number of the rrn operons per genome determine the rate of growth, are in accord with a theoretical analysis of the relationship between the specific growth rate and rrn operon expression [43]. This analysis led to the conclusion that M. tuberculosis should be capable of growing much faster than it actually does.

<table>
<thead>
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<th>Table 2</th>
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<tr>
<td>Comparison of genome sizes, the number of rrn operons per genome, the number of promoters per operon and generation times of representative mycobacteria.</td>
</tr>
<tr>
<td>Species</td>
</tr>
<tr>
<td>M. tuberculosis</td>
</tr>
<tr>
<td>M. marinum</td>
</tr>
<tr>
<td>M. simiae</td>
</tr>
<tr>
<td>M. leprae</td>
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<tr>
<td>M. chelonea</td>
</tr>
<tr>
<td>M. fortuitum</td>
</tr>
<tr>
<td>M. phele</td>
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<tr>
<td>M. smegmatis</td>
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</tbody>
</table>

$^{a}$ The genome size has been found to be strain dependent. Hence, both size and strain are cited. The sizes of the genomes of M. tuberculosis and M. leprae were obtained by sequencing (GenBank Accession Nos. AL123456 and AL450380 respectively). Other sizes were obtained from the kinetics of DNA renaturation [38] taking the size of the M. tuberculosis genome [28] as standard.

$^{b}$ All mycobacteria investigated to date have one operon (rrnA) located downstream from the murA gene [27]. Those species which have two rrn operons per genome have in addition to rrnA, a second operon located downstream from the tyrS gene [26].

$^{c}$ Generation time observed under optimal laboratory conditions. Except for M. leprae, which was grown in the footpads of mice, the data refer to shaken liquid cultures. M. marinum and M. chelonea were grown at 30 °C and the other mycobacteria at 37 °C.

<table>
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<th>Table 3</th>
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<tr>
<td>IS elements found in M. tuberculosis H37Rv, M. ulcerans and M. marinum.</td>
</tr>
<tr>
<td>Species</td>
</tr>
<tr>
<td>M. tuberculosis$^{a}$</td>
</tr>
<tr>
<td>M. ulcerans$^{b}$</td>
</tr>
<tr>
<td>M. marinum$^{c}$</td>
</tr>
</tbody>
</table>

$^{a}$ IS elements specified by Brosch et al., 2000 [34].

$^{b}$ IS elements reported by Stinear et al., 2000 [36].

$^{c}$ The genome of M. marinum has been tested by PCR amplification for the presence of IS sequences using primers that amplify IS 1245 and IS 1311 [42]; however, the complete complement has yet to be determined.

$^{d}$ Ref. [8].
with a defective inositol monophosphate phosphatase gene grows more slowly than the parental wild type [30].

Mobile genetic elements such as insertion sequences (IS) acquired by horizontal transfer are effective mutagenic agents. The restricted host range found for many IS elements [31,32] reflect the ecological environment of a bacterium. IS elements are known to be present in many mycobacterial genomes [33]. IS incorporated into the genome of M. tuberculosis are thought to have given rise to both chromosome rearrangements and deletions [34,35].

Although the three species M. marinum, M. ulcerans and M. tuberculosis are very closely related phylogenetically (see Table 3) each of them has a distinctive set of IS per genome. The different phenotypes of the three is consistent with the notion that the individual sets of IS have uniquely modified each genotype. The influence of IS elements on the properties of M. ulcerans have already been described [36,37]. M. marinum and M. tuberculosis each has a single rrr operon per genome and therefore each has a similar potential for rRNA synthesis. However, the presence of IS in the respective genomes have resulted in significant differences in properties. In particular it is proposed that the IS in the M. tuberculosis genome have attenuated cell metabolism leading to a much slower growth rate than is found for M. marinum.

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