The *dnaA* gene region of *Mycobacterium avium* and the autonomous replication activities of its 5' and 3' flanking regions

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A 3.9 kb DNA fragment containing the *dnaA* gene region of *Mycobacterium avium* was cloned and its nucleotide sequence was determined. Nucleotide sequence analyses indicated that this region encodes three genes in the order *rpmH* (ribosomal protein L34), *dnaA* (the putative initiator protein) and *dnaN* (the β subunit of DNA polymerase III). The intergenic regions between the *rpmH*–*dnaA* and *dnaA*–*dnaN* genes were found to contain several putative DnaA boxes, 9 nt long DnaA protein recognition sequences. A DNA fragment containing the 3' but not the 5' flanking region of the *M. avium dnaA* gene when cloned in *Escherichia coli* plasmids, which are otherwise non-replicative in mycobacteria, exhibited autonomous replication activity in *M. avium* but not in *Mycobacterium bovis* BCG and *Mycobacterium smegmatis*. The 5' flanking region of *dnaA*, on the other hand, exhibited autonomous replication activity in *M. bovis* BCG but not in *M. avium* and *M. smegmatis*. The implications of these results for the understanding of the *M. avium oriC* replication initiation process are discussed.

Keywords: *dnaA*, *oriC*, *Mycobacterium avium*, replication initiation

INTRODUCTION

Organisms of the *Mycobacterium avium*–intracellulare (MAC) complex are the most common cause of non-tuberculous mycobacterial infections outside the lung. Together they represent the single most common bacterial opportunistic infectious agent in AIDS, being diagnosed in up to 40% of AIDS patients. Furthermore, a recent report indicated that *M. avium* infection augments HIV-1 viral production (Wahl *et al.*, 1998). *M. avium* is a slow-growing mycobacterium with a typical generation time ranging from 10 to 12 h. Other mycobacteria exhibit varied growth rates with doubling times ranging from 2 to 3 h, e.g. *Mycobacterium smegmatis* and *Mycobacterium fortuitum*; to 22 to 24 h, e.g. members of the *Mycobacterium tuberculosis* complex (Wheeler & Ratledge, 1994).

The currently accepted model is that replication in eubacteria is initiated when the DnaA initiator protein

binds to DnaA boxes (9 nt DnaA protein recognition sequences) located in the origin of replication (*oriC*; Bramhill & Kornberg, 1988). The nucleotide sequence of these DnaA boxes is conserved and TTGTCCACA has been defined as their consensus sequence in Gram-positive bacteria with a high G+C content (see, for example, Fujita *et al.*, 1990; Qin *et al.*, 1997; Rajagopalan *et al.*, 1995b). The DnaA protein–box interactions result in a cascade of events, which culminate in replication initiation and DNA synthesis (Bramhill & Kornberg, 1988; Kornberg & Baker, 1991). The *dnaA* region in eubacteria, including *M. smegmatis*, *M. tuberculosis* and *Mycobacterium leprae*, shows both sequence and gene order conservation. The genes are present in the order 5'–*rrnA*–*rpmH*–*dnaA*–*dnaN*–*recF*–3' (Cole *et al.*, 1998; Qin *et al.*, 1999; Rajagopalan *et al.*, 1995a, b; Salazar *et al.*, 1996). In addition it has been shown in many bacteria that either the 3', 5' or both 3' and 5' flanking regions of the *dnaA* gene exhibit *oriC* activity, i.e. the presence of these sequences renders otherwise non-replicative plasmids capable of autonomous replication. In the case of *Bacillus subtilis* both the 5' and 3' flanking regions act as *oriC* (Moriya *et al.*, 1992). In *Pseudomonas putida* and *Pseudomonas*

Abbreviations: Km, kanamycin; OAD, oleic acid–albumin–dextrose.

The GenBank accession number for the sequence reported in this paper is U19185.

Table 1. Description of plasmids used in the study

Plasmid	Description	Source
Vectors used for sequencing		
pUC18	<i>E. coli</i> plasmid vector; Ap ^r	Lab stock
pMQ421	7 kb <i>Bam</i> HI fragment derived from the <i>M. avium</i> cosmid containing the <i>rpmH</i> and <i>dnaA</i> regions; Ap ^r	This study
pMQ422	Same as pMQ421 except that the 7 kb <i>Bam</i> HI fragment was cloned in the opposite orientation; Ap ^r	This study
pMQ423	2.1 kb <i>Eco</i> RI fragment derived from the <i>M. avium</i> cosmid containing <i>dnaA</i> and <i>dnaN</i> genes; Ap ^r	This study
pMQ431	1.7 kb <i>Sma</i> I– <i>Eco</i> RI fragment derived from pMQ421; Ap ^r	This study
pMQ432.6	1.3 kb <i>Sma</i> I fragment derived from pMQ434; Ap ^r	This study
pMQ432.8	1.0 kb <i>Sma</i> I fragment derived from pMQ434 (contains <i>rpmH</i>); Ap ^r	This study
pMQ433	2.2 kb <i>Kpn</i> I– <i>Bam</i> HI fragment derived from pMQ421; Ap ^r	This study
pMQ434	3 kb <i>Pst</i> I– <i>Bam</i> HI fragment derived from pMQ421, lacks the 5' flanking region of <i>rpmH</i> ; Ap ^r	This study
pMQ435	1.0 kb <i>Bam</i> HI large fragment derived from pMQ423; Ap ^r	This study
pMQ436	2 kb <i>Pst</i> I– <i>Eco</i> RI derived from pMQ421; Ap ^r	This study
pMQ437	0.7 kb <i>Sma</i> I– <i>Kpn</i> I derived from pMQ434; Ap ^r	This study
pMQ438	5 kb <i>Eco</i> RI fragment derived from pMQ422; Ap ^r	This study
pMQ439	3.9 kb <i>Pst</i> I– <i>Eco</i> RI fragment containing entire <i>dnaA</i> region of <i>M. avium</i> , i.e. <i>rpmH</i> , <i>dnaA</i> and <i>dnaN</i> genes and their intergenic regions; Ap ^r	This study
Vectors used for <i>oriC</i> activity		
pMQ456	1.3 kb <i>aph</i> gene was cloned into the <i>Pst</i> I site of pMQ423; Ap ^r , Km ^r	This study
pMQ457	1.3 kb <i>aph</i> gene was cloned into the <i>Pst</i> I site of pMQ434; Ap ^r , Km ^r	This study
pMQ459	1.3 kb <i>aph</i> gene was cloned into the <i>Bam</i> HI site of pMQ438; Ap ^r , Km ^r	This study
pMV206	<i>E. coli</i> –mycobacteria shuttle vector	Stover <i>et al.</i> (1991)
Vector used for DnaA overproduction		
pKYM5	1.5 kb <i>M. avium dnaA</i> gene cloned into the <i>Nde</i> I/ <i>Bam</i> HI sites of pET15b; Ap ^r	This study

* Ap, ampicillin; Km, kanamycin.

aeruginosa only the 5' flanking region acts as *oriC* (Yee & Smith, 1990) while in *Streptomyces* spp. (Zakrzewska-Czerwinka & Schrepf, 1992), *M. smegmatis* (Qin *et al.*, 1997; Rajagopalan *et al.*, 1995b; Salazar *et al.*, 1996), *M. bovis* and *M. tuberculosis* (Qin *et al.*, 1999) only the 3' flanking region functions as *oriC*. The genetic elements responsible for the replication process in *M. avium*, specifically how it is initiated and regulated, are not known. In view of the clinical significance of this organism it is important to identify and characterize the key players involved in *M. avium* replication initiation.

In this study we have cloned and determined the gene order of the *dnaA* region of *M. avium* and examined whether the *dnaA* gene flanking regions exhibit *oriC* activity in *M. avium* (Rajagopalan *et al.*, 1995b). We show that the gene order of the *dnaA* region of *M. avium* is similar to that reported for other bacteria, and the 3' but not the 5' flanking region of *dnaA*, when cloned in

non-replicative plasmids, exhibited *oriC* activity in *M. avium*. Interestingly the 5' flanking region of *M. avium* also exhibited autonomous replication activity but only in *M. bovis* BCG. The implications of these results for understanding *M. avium* replication are discussed.

METHODS

Bacterial strains and growth media. *M. avium* MAC104 was obtained from Dr Luiz Bermudez, Kuzell Institute, San Francisco, USA; *M. intracellulare* mc²76 was from Dr Raul Barletta, University of Nebraska, Lincoln, USA (Foley-Thomas *et al.*, 1995); *M. smegmatis* mc²155 was from Dr W. R. Jacobs, Jr, Albert Einstein College of Medicine, Bronx, New York, USA (Snapper *et al.*, 1990); *M. bovis* BCG was from Dr Y. Taniguchi, University of Occupational and Environmental Health, Kitakyushu, Japan (Goto *et al.*, 1991);

Escherichia coli BL21(DE3) lysogen was from Dr Studier, Brookhaven National Laboratory, New York, USA (Studier *et al.*, 1990); and *E. coli* DH5 α was a laboratory stock. *E. coli* was grown in Luria–Bertani (LB) medium whereas mycobacteria were grown in Middlebrook 7H9 medium supplemented with oleic acid–albumin–dextrose (OAD) as described by Jacobs *et al.* (1991) and Rajagopalan *et al.* (1995b). *E. coli* transformants were selected on LB agar containing either ampicillin or kanamycin (Km) at a final concentration of 50 $\mu\text{g ml}^{-1}$. Mycobacterial transformants were selected on Middlebrook 7H10 agar medium supplemented with OAD and Km at a final concentration of 25 $\mu\text{g ml}^{-1}$ (Qin *et al.*, 1997; Rajagopalan *et al.*, 1995b).

Recombinant DNA techniques. The pYUB18-derived *M. avium* cosmid libraries were obtained from Drs Lisa Pascopella and Raul Barletta. All plasmids, unless otherwise stated, were derived from pUC18 and are described in Table 1. For selecting recombinants/transformants in mycobacteria, a 1.3 kb DNA fragment containing the *aph* gene for Km resistance was cloned into the multiple cloning site (Rajagopalan *et al.*, 1995b). Plasmid DNA from mycobacterial transformants was recovered into *E. coli* using the zirconia-based bead-beating technique (Qin *et al.*, 1997; Madiraju *et al.*, 1999). Oligonucleotides used in the study were synthesized in a Pharmacia Gene Assembler Special (Pharmacia) or were purchased from Operon Technologies. Standard molecular biology techniques such as plasmid analyses, labelling of DNA and Southern hybridization techniques were as described by Sambrook *et al.* (1989). DNA sequencing was performed either in a Pharmacia ALF DNA sequencer using the Pharmacia autocycle sequencing kit and protocol or in a LICOR DNA sequencer at McConnel Research Laboratories, San Diego, USA. Both forward and reverse sequencing primers were used as needed to determine the nucleotide sequence of both DNA strands.

Transformation conditions. Exponentially growing cultures of *M. avium* (approximate OD₆₀₀ of 0.5; Shimadzu UV–visible UV-1201 spectrophotometer, 1 cm path length) were diluted 100-fold in Middlebrook 7H9 broth supplemented with OAD in tissue culture flasks. Cultures were incubated at 37 °C for 8–10 d until they reached an OD₆₀₀ of 0.5. One day prior to harvesting the culture, 15% (w/v) glycine stock was added to give a final concentration of 1.5%. The cultures were harvested, washed twice at room temperature with an equal volume of 10% (v/v) glycerol, then resuspended in approximately 0.01 vols of the starting culture and used immediately for electroporation with the test plasmids. Electroporation was carried out at room temperature at 2.5 kV, 25 μF and 1000 Ω . Approximately 0.2–0.3 μg plasmid DNA was used in the transformation experiments. Following electroporation, 0.9 ml Middlebrook 7H9 broth supplemented with OAD was added, cultures were incubated overnight at 37 °C, appropriate dilutions were spread on antibiotic plates and incubated at 37 °C for 2–3 weeks. The transformation efficiency with *M. avium* MAC104 under current experimental conditions was in the range of 10^2 – 10^3 transformants (μg input plasmid DNA)⁻¹ and was relatively poor compared to other mycobacterial strains (see Results and Discussion). The use of greater amounts of plasmid DNA ($\sim 1 \mu\text{g}$ and more), or increasing the temperature of electroporation to 37 °C (Wards & Collins, 1996), or the addition of D-norleucine and *m*-fluoro-D-L-phenylalanine prior to harvesting cultures (D. L. Lakey, C.

Hager, D. S. Kernodel & K. M. Edwards, unpublished data) did not result in any increase in the transformation efficiency.

Transformation conditions for *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* were as described by Qin *et al.* (1999, 1997) and Wards & Collins (1996). Since the *oriC* regions of *M. bovis* BCG and *M. tuberculosis* differ by only one nucleotide and *oriC* plasmids from each function in the other, the mechanism of replication initiation in *M. tuberculosis* and *M. bovis* BCG is believed to be very similar (Qin *et al.*, 1999). Consequently, to avoid any potential risk to the experimenters all transformation experiments involving *M. tuberculosis oriC* plasmids were carried out in *M. bovis* BCG.

oriC activity. *oriC* activity was defined as the ability of *dnaA* region fragments from *M. avium* to render *E. coli* plasmids, which were otherwise unable to replicate, capable of autonomous replication in *M. avium*. The results are expressed as the total number of Km^r transformants obtained (μg input DNA)⁻¹ (Qin *et al.*, 1997; Rajagopalan *et al.*, 1995b). Since our results showed that the 5' and 3' flanking regions of the *M. avium dnaA* gene exhibited different host-specific autonomous replication activities, to distinguish between these we refer to autonomous replication activity of *M. avium* plasmids in *M. avium* hosts as *oriC* activity and replication in other mycobacterial hosts as autonomous replication activity.

Cloning of the *dnaA* region. All plasmid manipulations were carried out in *E. coli* (Sambrook *et al.*, 1989). MVM37, an oligonucleotide primer specific to the *M. avium dnaA* gene (5'-AACAAGACCCGTCATCGACCGGTCTGCGCCGAG-3'; Rajagopalan *et al.*, 1995a) was used to screen *M. avium* cosmid DNA libraries by colony hybridization (Sambrook *et al.*, 1989). The cosmids from the positive clones were confirmed by Southern hybridization, probing individually with DNA fragments specific to the *rpmH*, *dnaA* and *dnaN* genes. Based on these data pUC18-based plasmids with inserts containing the 3.0 kb *rpmH* and *dnaA* genes (pMQ434) and 2.1 kb *dnaA* and *dnaN* genes (pMQ423) and various subclones were constructed and used to determine the nucleotide sequence of both strands of the inserts (see Fig. 1). Typically 400–500 bases of sequence per sample were obtained with the Pharmacia ALF DNA sequencer and approximately 750–1150 bases of sequence with the LICOR machine. Nucleotide sequence data were analysed by DNA Strider 1.3 and Gene Jockey programs. Once the nucleotide sequence was confirmed, the *dnaA* region fragments in both pMQ434 and pMQ423 were joined to produce a 3.9 kb DNA fragment containing the *rpmH*, *dnaA* and *dnaN* genes and their intergenic regions.

Construction of plasmids overexpressing *M. avium dnaA*. The *M. avium dnaA* coding region was amplified by PCR using the primers 5'-CGCCTGCAGCATATGGCCGATG-ACCCCGGTTC-3' and 5'-CGCGGATCCCTAGCGCTTG-AGCG-3', and cloned into the *NdeI/BamHI* sites of the pET15b vector (Novagen). The resultant vector, designated pKYM5 and containing the *M. avium dnaA* gene under T7 transcription and translational signals, was introduced into *E. coli* BL21(DE3) (Studier *et al.*, 1990). To detect the *M. avium* DnaA protein, *E. coli* cultures containing the plasmids were grown in LB broth to an OD₆₀₀ of 0.8 and DnaA overproduction was then induced by the addition of IPTG for 3 h. Cultures were harvested, both soluble and insoluble extracts were prepared (Sambrook *et al.*, 1989), and proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining (Laemmli, 1970).

Fig. 1. The *dnaA* region of *M. avium*. A physical map of the 7 kb DNA fragment containing the *M. avium* *dnaA* gene region is shown. Single letter codes indicate restriction endonuclease sites: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sma*I. The 3.9 kb DNA fragment containing the *rpmH*, *dnaA* and *dnaN* genes is marked. The nucleotide sequence of both strands of the 3.9 kb DNA fragment was analysed by the DNA Strider 1.3 program to deduce the location of the *rpmH*, *dnaA* and *dnaN* genes. Arrowheads below the genes indicate the direction of transcription. The names of the plasmid constructs used to determine the nucleotide sequence of the *dnaA* region are shown on the right and the corresponding DNA fragments are shown on the left. Note that pMQ421 lacks *dnaN* whereas pMQ423 lacks *rpmH*. Various subclones were derived from these plasmids (see Table 1) and were subsequently used to determine the nucleotide sequence in either the Pharmacia Automated Fluorescent DNA sequencer or the LICOR DNA sequencer.

RESULTS AND DISCUSSION

The *dnaA* region and sequence analysis

Fig. 1 shows the various plasmid constructs used to determine the nucleotide sequence of the 3.9 kb *dnaA* region of *M. avium*. A BLAST search (Altschul *et al.*, 1990) revealed homologies with the *rpmH*, *dnaA* and *dnaN* genes of several bacteria, including *M. tuberculosis* and *M. smegmatis* (Cole *et al.*, 1998; Qin *et al.*, 1999; Rajagopalan *et al.*, 1995b; Salazar *et al.*, 1996). Three ORFs, referred to as *orf1*, *orf2* and *orf3*, were identified. The start codons for *orf1*, *orf2* and *orf3* are predicted to be GTG, TTG and ATG, respectively. *orf1* is smaller than *orf2* and *orf3*, and the direction of transcription of *orf1* appears to be opposite to that of *orf2*. The putative termination codon in *orf3* was not detected.

orf1 corresponds to nucleotide positions 480–340 of the bottom strand. It is predicted to encode an RpmH homologue of 47 residues with a molecular mass of 5.3 kDa. Six nucleotides upstream of the predicted start codon, GTG, is AGAAGG, a sequence resembling that of a potential ribosome binding sequence. The predicted protein exhibits significant homology with the RpmH

protein of other bacteria, including *M. tuberculosis* and *M. smegmatis* (data not shown). *orf2* corresponds to nucleotide positions 1084–2610, is the largest of the three ORFs and is predicted to encode the DnaA homologue. Eight nucleotides upstream of the predicted start codon, TTG, is a potential ribosome binding sequence, AGGGAG. The predicted protein is 509 residues in length and has a molecular mass of 56.7 kDa. The deduced amino acid sequence of the *M. avium* *dnaA* gene product is 86 and 55% identical to the corresponding proteins of *M. tuberculosis* and *M. smegmatis*, respectively (data not shown). *orf3* corresponds to nucleotide positions 3179–end and is predicted to encode the *dnaN* homologue. Five nucleotides upstream of the ATG start codon is the putative ribosome binding sequence, GAAGGG. The predicted protein shows significant homology with the DnaN protein of other bacteria, including mycobacteria (data not shown).

The *rpmH*–*dnaA* and *dnaA*–*dnaN* intergenic regions

One of the characteristic features of eubacterial *oriC* is the presence of several DnaA-box-like sequences. The *rpmH*–*dnaA* and *dnaA*–*dnaN* intergenic regions of *M. avium* were found to contain 14 and 16 DnaA-box-like sequences with one to three mismatches to the consensus sequence TT(G/C)TCCACA, respectively (see Fig. 2; Qin *et al.*, 1999, 1997; Rajagopalan *et al.*, 1995b). These are clustered close to the *dnaA* gene itself with 10 of the 14 DnaA boxes in the *rpmH*–*dnaA* intergenic region located towards the 5' end of *dnaA*, and 11 of the 16 DnaA boxes in the *dnaA*–*dnaN* intergenic region towards its 3' end. Nucleotide sequence comparisons revealed that the *M. avium* *rpmH*–*dnaA* intergenic region is 63 and 45% identical to that of the corresponding regions of *M. tuberculosis* and *M. smegmatis*, respectively, whereas the *dnaA*–*dnaN* intergenic region is 62 and 50% homologous, respectively. Many of the designated DnaA boxes of *M. avium* show significant sequence homology to the corresponding position in *M. tuberculosis*, suggesting that the equivalent DnaA boxes in both organisms are positionally conserved (Fig. 2a and b). In contrast, the *M. smegmatis* sequence does not show DnaA boxes at many of these conserved positions (Fig. 2a and b). This suggests that the architectural composition of *oriC* of *M. smegmatis* differs from *M. avium* and *M. tuberculosis* (Fig. 2).

Localization of *oriC* activity

Plasmid constructs containing either the *rpmH*–*dnaA* intergenic region (pMQ457 and pMQ459) or the *dnaA*–*dnaN* intergenic region (pMQ456) were tested for their ability to exhibit *oriC* activity in *M. avium*. *M. avium* transformed with pMQ456 produced Km^r colonies but pMQ457 and pMQ459 produced none (data not shown). The observed transformation frequency with pMQ456 was between 10² and 10³ transformants (μg input DNA)^{−1} which is comparable to that observed with the shuttle plasmid pMV206 (data not shown). To test whether the observed Km resistance was due to stable

Fig. 2. For legend see p. 2918.

extrachromosomal replication of the pMQ456, four independent transformants were streaked for single colony isolation and one independent colony from each pool was grown. Their plasmid DNA was recovered into *E. coli* (Qin *et al.*, 1997) and subsequently analysed by restriction digestion. Restriction digestion patterns of the recovered plasmids were indistinguishable from that of the input plasmid used (Fig. 3), suggesting that little

or no changes to the plasmids had occurred during growth and that these plasmids were indeed replicating as extrachromosomal elements in *M. avium*. The inability of the *rpmH*–*dnaA* intergenic region to support *oriC* activity, despite the presence of multiple DnaA boxes, indicates that other factors or features are also necessary for it to exhibit *oriC* activity in *M. avium* (see below). Alternatively, this region could be involved in

Fig. 2. Nucleotide sequence comparisons of the *rpmH-dnaA* (a) and *dnaA-dnaN* (b) intergenic regions. Nucleotide sequence of the intergenic regions of the *rpmH-dnaA* and *dnaA-dnaN* of *M. avium*, *M. tuberculosis* and *M. smegmatis* were aligned using CLUSTAL W multialignment and the data are presented by using the SEQVU 1.0.1 program. Presumptive DnaA boxes of *M. avium* with one to three mismatches to the consensus sequence TTG/CTCCACA (Qin *et al.*, 1997; Rajagopalan *et al.*, 1995b) are shown as arrows above the sequence. The direction of the arrows indicates the orientation of the DnaA boxes. Identical nucleotide bases are shown in shaded boxes. A dash indicates a gap created for alignment. Nucleotides are numbered to the left of the sequence. DnaA boxes of *M. avium* that showed either zero or one mismatch to that of the corresponding *M. tuberculosis* sequence are marked by appropriate numbers adjacent to the arrows. Mac, *M. avium* 104 (U19185); Mtb, *M. tuberculosis* H37Rv (U38891); Msmeg, *M. smegmatis* mc²155 (U17833).

Fig. 3. Restriction digestion pattern analysis of *M. avium* *oriC* plasmids recovered from Km^r transformants. Four independent transformants of *M. avium* were individually propagated and plasmid DNA was recovered into *E. coli*. Plasmid DNA was purified, restricted with *Pst*I, electrophoretically separated in agarose gels, visualized by ethidium bromide staining and photographed. As a control, the input plasmid pMQ456 was digested with *Pst*I and processed as described above. Lanes: 1, 1 kb ladder; 2, input plasmid pMQ456 DNA digested with *Pst*I; 3–6, pMQ456 DNA recovered from *M. avium* transformants and digested with *Pst*I.

the regulation of expression of its immediate flanking genes (Atlung *et al.*, 1987; Kornberg & Baker, 1991; Skarstad & Boye, 1994).

M. avium* *oriC* does not function in *M. tuberculosis* and *M. smegmatis

Replication origins from bacterial members belonging to the same family or closely related family members often appear to function in each other (Kornberg & Baker, 1991; Skarstad & Boye, 1994; Skovgaard & Hansen, 1987; Smith *et al.*, 1991; Yee & Smith, 1990; Zakrzewska-Czerwinka & Schrepf, 1992; Zyskind & Smith, 1986). These data led to the suggestion that the mechanisms involved in the initiation of replication in members belonging to the same family are similar. Mycobacteria appear to be an exception to this rule because the *M. smegmatis* *oriC* does not function in *M. tuberculosis* or *M. bovis* BCG and vice versa (Qin *et al.*, 1999). To test whether the *M. avium* *oriC* functions in other mycobacteria, we electroporated *M. bovis* BCG and *M. smegmatis* with the *M. avium* *oriC* plasmid (pMQ456) and vice versa. As controls, both *M. bovis* BCG and *M. smegmatis* were also electroporated with pMV206, an *E. coli*–mycobacteria shuttle vector. Km^r colonies were obtained only with the shuttle vector in each case and the transformation frequencies were in the range of 10^4 (for *M. bovis* BCG) to 10^5 (for *M. smegmatis*) transformants (μg input DNA) $^{-1}$. Together these data indicate that *M. avium* *oriC* is specific to *M. avium*. This species specificity of replication origins, despite their conserved sequence homology, suggests that the regulatory mechanisms involving the initiation of replication might be different in each of these species.

Fig. 4. Restriction digestion analysis of pMQ457 recovered from *M. bovis* BCG transformants. Four independent transformants of *M. bovis* BCG were propagated on Middlebrook 7H10 agar plates supplemented with OAD and Km . Plasmid DNA was recovered into *E. coli*, purified, restricted with *Eco*RI and analysed as described in the legend to Fig. 3. Lanes: 1, 1 kb ladder; 2–5, pMQ457 DNA recovered from *M. bovis* BCG transformants and digested with *Eco*RI; 6, input plasmid pMQ457 DNA digested with *Eco*RI.

Autonomous replication activity of *M. avium* DNA fragments containing the *rpmH*–*dnaA* intergenic region

We have speculated that the *M. avium* replication origins might have evolved from *B. subtilis*-like organisms (Moriya *et al.*, 1992) that selectively utilize the *dnaA*–*dnaN* intergenic region as their primary *oriC*. If this is the case the *rpmH*–*dnaA* intergenic region may be a vestigial replication origin or alternatively it could be a silent replication origin whose activity can be unmasked under specific growth conditions, e.g. in the absence of specific inhibitors. To test this prediction, pMQ457 (containing the *rpmH*–*dnaA* intergenic region) was transformed into both *M. smegmatis* and *M. bovis* BCG. *M. bovis* BCG transformed with pMQ457 produced Km^r transformants but *M. smegmatis* did not (data not shown; see below). The *M. bovis* BCG transformants appeared 2 weeks later than those with the shuttle vector pMV206. The transformation frequency was in the range of 10^4 (μg input DNA) $^{-1}$ and was similar to that obtained with the shuttle vector pMV206. The recovered and input pMQ457 DNA showed identical restriction digestion patterns (Fig. 4), indicating that pMQ457 was replicating in *M. bovis* BCG as stable extrachromosomal elements (data not shown). Reciprocal experiments using *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis* plasmid constructs containing their *rpmH*–*dnaA* intergenic region in *M. avium* hosts did not produce any Km^r transformants (data not shown). Since the transformation efficiency of *M. avium* strains is two to three orders of magnitude less

Fig. 5. Overexpression of recombinant DnaA protein. BL21(DE3) cultures harbouring pKYM5 were grown to an OD_{600} of 0.8 and DnaA production was induced by the addition of 1 mM IPTG. Total lysates were prepared and centrifuged to separate the soluble and insoluble fractions. Proteins were resolved by 10% SDS-PAGE and visualized by Coomassie blue staining. Lanes: 1, protein molecular mass markers; 2, uninduced culture; 3, induced culture – soluble proteins; 4, induced culture – insoluble proteins. The arrowhead indicates the position of DnaA.

than the other mycobacterial strains, it is possible that the lack of autonomous replication activity of those plasmids in *M. avium* may be merely due to poor transformation efficiency of *M. avium* or could be a property specific to *M. avium*. Obviously improved transformation conditions with *M. avium* strains would help resolve this issue.

The host-specific activities of *M. avium* DNA fragments containing the *rpmH*–*dnaA* and *dnaA*–*dnaN* intergenic regions we have demonstrated are distinctly different from those reported for other bacteria. The autonomous replication of pMQ457 in *M. bovis* BCG indicates that the *M. avium* *rpmH*–*dnaA* intergenic region sequence is able to utilize the *M. bovis* BCG replication machinery to initiate and replicate the pMQ457. Considering the significant sequence similarities in the intergenic region of these two organisms (Fig. 2) as well as the *dnaA* gene itself this is not totally unexpected. However, it appears paradoxical that pMQ457 failed to replicate in *M. avium*. One possible explanation is that the *dnaA*–*dnaN* intergenic region of *M. avium*, which acts as a primary *oriC*, could exert an inhibitory effect on the *oriC* activity originating at the *rpmH*–*dnaA* intergenic region. In this scenario the *rpmH*–*dnaA* intergenic region of *M. avium*

would serve as an alternative replication origin and exhibit *oriC* activity *in vivo* only under conditions where replication at the primary *oriC* is inhibited or when the putative inhibitory regulators are switched off. The ability of *M. avium* to selectively utilize one or other of the replication origins might provide a selective advantage if activity from one were suppressed. This could allow it to continue to grow and divide for longer periods of time in a hostile environment.

Overproduction of DnaA of *M. avium*

Since the interaction between the DnaA protein and DnaA boxes is believed to be the essential prerequisite for initiation of replication (Kornberg & Baker, 1991), we have begun to investigate this process in *M. avium*. We have overproduced *M. avium* DnaA in *E. coli*. Unfortunately, most of the DnaA was in inclusion bodies (Fig. 5). We are pursuing a number of strategies to obtain soluble protein but until it can be obtained in a sufficient quantity in its native form it will not be possible to investigate its binding properties any further.

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