Mycobacterium smegmatis DNA gyrase: cloning and overexpression in Escherichia coli

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INTRODUCTION

DNA topoisomerases play a crucial role in a variety of cellular processes by maintaining the chromosomal superhelical density. DNA gyrase is a bacterial type II DNA topoisomerase which catalyses negative supercoiling of DNA. Although all known topoisomerases have the ability to relax negatively supercoiled DNA, introduction of negative supercoils into DNA is the unique property of DNA gyrase (Cozzarelli, 1980; Reece & Maxwell, 1991a). The enzyme from Escherichia coli, discovered by Gellert et al. (1976) serves as a prototype. It is encoded by two genes, gyrA and gyrB, producing A and B subunits of molecular mass 97 and 90 kDa, respectively. The functional enzyme is a tetrameric (A₂B₂) protein, having supercoiling-relaxation, catenation-decatenation and knotting-unknotting activities (Wang, 1985; Reece & Maxwell, 1991a). The major role assigned to the GyrA subunit is the transient breakage and reunion of DNA, while the GyrB subunit is concerned with ATP hydrolysis providing energy required for the supercoiling reaction (Reece & Maxwell, 1991a and references therein). The enzyme is a target for two groups of compounds, quinolones and coumarins. Two plasmid-encoded protein poisons (Vizan et al., 1991; Bernard & Couturier, 1992), and possibly some new classes of natural compounds (Osborne et al., 1990; Nakada et al., 1993), have also been reported to inhibit the enzyme. The quinolones act on the GyrA subunit–DNA complex, whereas coumarins act on the GyrB subunit. Similarly, the F plasmid-encoded CcdB (LetD) protein inhibits gyrase activity by complexing with GyrA (Bernard & Couturier, 1992), while microcin B17 (encoded by plasmid MCC B17) inhibits gyrase by interacting with GyrB (Vizan et al., 1991).

The gyrA and gyrB genes from E. coli have been cloned, sequenced (Yamagishi et al., 1986; Adachi et al., 1987; Swanberg & Wang, 1987), expressed (Hallet et al., 1990) and extensively characterized. Since then, there have been reports on the cloning of DNA gyrase genes from several bacterial species primarily to study the molecular basis of quinolone resistance. The genes have been cloned from Bacillus subtilis (Lampe & Bott, 1984; Moriya et al., 1985), Streptomyces sphaeroides (Thiara et al., 1988), Streptomyces coelicolor (EMBL accession no. L27063), Staphylococcus aureus (Brockbank & Barth, 1993), Mycoplasma pneumoniae (Colman et al., 1990), Caulobacter crescentus (Rizzo et al., 1993) and Mycobacterium tuberculosis (Madhusudan et al., 1994a, b; Takiff et al., 1994). As is the
case for *E. coli*, all these bacteria have the enzyme encoded by two genes, *gyrA* and *gyrB*.

The genus *Mycobacterium* comprises several important species of public health concern. Apart from *M. tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy, respectively, many species cause disease in mammals under a variety of opportunistic situations (Collins, 1993). The resurgence of tuberculosis in the New World and the emergence of multiple-drug-resistant, highly virulent *M. tuberculosis* clinical strains have raised global alarm (Kaufmann & van Embden, 1993; Young & Cole, 1993). The rapid increase in mortality and morbidity due to tuberculosis infections warrants immediate attempts to develop new antimycobacterials. Detailed molecular analysis of DNA gyrase from *Mycobacterium* thus gains importance. *Mycobacterium smegmatis*, a non-pathogenic and fast-growing species, wherein gene transfer and genetic manipulation systems have been developed, serves as a model system for studying the mechanism of drug resistance (Snapper et al., 1990; Young & Cole, 1993). Here, we report the cloning, analysis of the primary structure, and the organization of DNA gyrase genes from *M. smegmatis*. The genes were cloned into T7 expression vectors and expressed in *E. coli*. The functional enzyme could be reconstituted from independently expressed subunits to obtain DNA supercoiling activity which is unique to DNA gyrase.

**METHODS**

**Bacterial strains and plasmids.** *M. smegmatis* SN2 (laboratory stock) was propagated on modified Youmans & Karlson’s medium (Nagaraja & Gopinathan, 1980). *E. coli* DH10B was used for all cloning experiments. DNA gyrase temperature-sensitive mutants *E. coli* NH647 (*gyrA*<sup>ts</sup>) and *E. coli* N4177 (*gyrB*<sup>ts</sup> *cou*<sup>ts</sup>) were obtained from Nicholas R. Cozzarelli (University of California, Berkeley, CA, USA) and Martin Gellert (National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD, USA), respectively. Plasmids pMN13R and pMN6R have been described earlier (Madhusudan et al., 1994a, b). The plasmid pJW312-SalI used in the purification of *E. coli* topoisomerase I was a generous gift from James C. Wang, Harvard University, Cambridge, MA, USA. A brief description of the plasmids and the bacterial strains is given in Table 1.

**Materials.** Restriction and modifying enzymes were either from Boehringer Mannheim or New England Biolabs and used according to the suppliers’ recommendations. Novobiocin was from Boehringer Mannheim. Oligodeoxynucleotides were obtained from BioServe Biotechnologies.

**Southern analysis and isolation of gyrase genes.** The chromosomal DNA was isolated from *M. smegmatis* SN2 as described by Madhusudan et al. (1994a) and digested with BglII, *EcoRI*, *Asp718*, *PstI* and *StuI*. The DNA digests were electrophoresed on 0.8% agarose gel and blotted onto Genescan plus membrane by alkali transfer (Reed & Mann, 1985). The genomic blot was hybridized to 12 kb *EcoRI*–*HpaI* fragment from plasmid pMN13R containing *gyrB* of *M. tuberculosis* in 6 × SSC, 1% (w/v) SDS, 5 × Denhardt’s solution and 500 μg yeast total RNA ml<sup>−1</sup> at 65 °C. The probe was labelled to high specific activity using Klenow fragment of DNA polymerase I-mediated extension of mixed random hexanucleotides annealed to the probe, in the presence of [α-<sup>32</sup>P]CTP (> 3000 Ci mmol<sup>−1</sup>; 111 TBq mmol<sup>−1</sup>). The blot was then washed three times with 0.1 × SSC plus 0.1% SDS at 68 °C and autoradiographed. The DNA fragments corresponding to 4.5 kb *Asp718* and 4.0 kb *BglII* were purified from agarose gel by electroelution and cloned into pUC19 at the compatible sites. The resultant plasmids were sequenced using dideoxynucleotide chain-termination method (Sanger et al.,

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<th>Strain/plasmid</th>
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<tr>
<td><em>E. coli</em> NH647</td>
<td><em>gyrA</em>&lt;sup&gt;ts&lt;/sup&gt;</td>
<td>N. R. Cozzarelli</td>
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<td><em>E. coli</em> N4177</td>
<td><em>gyrB</em>&lt;sup&gt;ts&lt;/sup&gt; <em>cou</em>&lt;sup&gt;ts&lt;/sup&gt;</td>
<td>M. Gellert</td>
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<td>pJW312-SalI</td>
<td><em>E. coli</em> topA under the control of Plac</td>
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<td>pMK90</td>
<td><em>E. coli</em> <em>gyrA</em> in pCK16</td>
<td>Mizuuchi et al. (1984)</td>
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<td>pMK47</td>
<td><em>E. coli</em> <em>gyrB</em> in pCK16</td>
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<td>pMN13R</td>
<td><em>M. tuberculosis</em> <em>gyrB</em> in pBR322</td>
<td>Madhusudan et al. (1994b)</td>
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<td>pMN6R</td>
<td><em>M. tuberculosis</em> <em>gyrA</em> in pUC19</td>
<td>Madhusudan et al. (1994b)</td>
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RESULTS

Cloning of M. smegmatis DNA gyrase genes

DNA gyrase genes were previously cloned from M. tuberculosis (Madhusudan et al., 1994a, b). The EcoRI–HpAI (1.2 kb) fragment encompassing two-thirds of the gyrB gene from plasmid pMN138 (Madhusudan et al., 1994b) was used as the probe to isolate gyrB gene from M. smegmatis. Genomic DNA of M. smegmatis was digested with the restriction enzymes BglII, AscI18, EcoRI, PstI and SmaI and subjected to hybridization analysis (Fig. 1a). The same blot was also probed with the gyrA gene of M. tuberculosis (1.0 kb Mini–SmaI fragment from pMN6R; Madhusudan et al., 1994b). It was found that both the 4.5 kb Asp718 and the 4.0 kb BglII fragments hybridized to this probe (not shown), suggesting that these two fragments are contiguous. Both the fragments were eluted from the agarose gel and ligated to pUC19 linearized with either Asp718 or BamHI. The DNA was used to transform E. coli DH10B and these size-selected enriched libraries were screened by colony hybridization with the same probe used in the genomic Southern blot analysis. The recombinant plasmids, pMN6A (carrying the Asp718 fragment) and pMN18B (carrying the BglII fragment) were then examined by restriction mapping to establish the overlapping nature of the DNA inserts (Fig. 1b). The sequencing strategy is also presented in Fig. 1(b). The ordered clones were generated by using restriction enzymes as well as exonuclease III-mediated unidirectional deletions.

Nucleotide sequence analysis for reading frames

The 5119 bp nucleotide sequence between a unique Mael and the second SmaI site (Fig. 1b) is presented with the deduced amino acid sequence in Fig. 2. Sequence analysis revealed the presence of two long ORFs on the top strand, shown in Fig. 2, and three on the complementary strand. The two forward ORFs, ORF675 and ORF854, share a high degree of similarity with the known DNA gyrase B and A subunits, respectively. ORF675 has the Shine–Dalgarno sequence AGGAG at nucleotide position 2167. Thus, DNA gyrase genes are also conserved in the case of M. tuberculosis (Fig. 2). Recently, Revel et al. (1994) have reported the sequence of the quinolone-resistance-determining region of M. smegmatis gyrA. This sequence is identical to the nucleotides between positions 2442 and 2549 in Fig. 2. The G + C content of the gyrB and gyrA genes is 65 mol% and conforms to the average G + C content of the genome. The codon usage pattern was also different from that of other mycobacterial genes. Analysis of the complementary strand showed three major ORFs. The fasta (Pearson & Lipman, 1988) search did not show significant

DNA supercoiling assay. Cell extracts from overexpressing strains were prepared by sonication in buffer A (20 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.2 M KCl, 10%, v/v, glycerol) and the S100 superantigen was subjected to polyvinyl P precipitation (0.5%), followed by ammonium sulfate (70% saturation) fractionation. The pellets were dissolved in buffer A and dialysed against the same buffer.

For the enzyme assay, GyrA and GyrB subunits were mixed in equal amounts (as estimated by SDS-PAGE analysis) and incubated on ice for 30 min. Form IV DNA, prepared by treatment of supercoiled pUC19 and purified E. coli topoisomerase I (Lynn & Wang, 1989), was used as the substrate for DNA gyrase. DNA supercoiling assay was carried out as described by Mitsuuchi et al. (1978). The reaction mixture (20 μl) containing 35 mM Tris/HCl, pH 7.6, 24 mM KCl, 6 mM MgCl₂, 0.14 mM EDTA, 5 mM DTT, 1:8 M spermine hydrochloride, 90 μg yeast tRNA ml⁻¹, 6.5%, v/v, glycerol, 50 μg BSA ml⁻¹, 1:4 mM ATP and 1 μg relaxed pUC19 was incubated with either of the subunits (500 ng each) or the reconstituted enzyme (100–500 ng) at 37° C for 30 min. The reaction was stopped by adding 4 μl 0.6% SDS, 0.2% bromophenol blue, 0.2% xylene cyanol FF, and the samples were electrophoresed in 10% (w/v) agarose gel and photographed.
similarity of ORF617 (3075–4928 bp) or ORF309 (2145–3074 bp) to any known sequence from the database. However, ORF711 (40–2175 bp) shared some similarity with the hypothetical 80.5 kDa protein from *Halofex* spp. (Holmes & Dyall-Smith, 1991; Swiss-Prot accession no. P21562). Interestingly, this hypothetical polypeptide sequence was also deduced from the complementary strand of the *gyrB* gene. Such long reading frames were not found on the complementary strand of gyrase genes from *M. tuberculosis* and *S. sphaeroides*. These reading frames, however, showed a low bias to G+C at the third position of the codon.

**Comparison of the amino acid and DNA sequences of DNA gyrase genes**

DNA gyrase genes characterized so far from diverse species show considerable sequence similarity. To determine the degree of conservation of gyrase genes in *M. smegmatis*, we resorted to pairwise comparisons. Dot matrix analysis of *M. smegmatis* gyrase genes with that of *E. coli* and *M. tuberculosis* is shown in Fig. 3. This analysis reveals that the proteins from the two species of *Mycobacteria* are nearly identical except for small regions of sequence divergence. In contrast, the *E. coli* sequence has a large insertion closer to the carboxy-terminus of GyrB (Fig. 3a) and another small one in the middle of GyrA (Fig. 3b). The carboxy-terminal region of GyrA shows some repeated sequences. These are seen as off-diagonal matches in the Harr plot (Fig. 3b). The carboxy-terminal domain of *E. coli* GyrA has been shown to be a DNA-binding protein (Reece & Maxwell, 1991b). The pair-wise comparisons using *gap* (UWGCG software) showed that the *gyrB* gene of *M. smegmatis* shares 86 and 53% identity with that of *M. tuberculosis* and *E. coli*, while the *gyrA* gene shares 88.4 and 45% identity, respectively, at the amino acid level. The genes shared high similarity with that of *M. tuberculosis*, also at the nucleotide level.

**Complementation studies**

*E. coli* DNA gyrase temperature-sensitive mutant strains, *E. coli* NH647 (*gyrA*ts) and *E. coli* N4177 (*gyrB*ts cou) which do not grow at 42 °C were transformed with *M.
DNA gyrase from Mycobacterium smegmatis

**Fig. 2.** Nucleotide sequence of *M. smegmatis* DNA gyrase genes with the flanking regions. The deduced amino acid sequences of two major reading frames are given below the nucleotide sequence. Shine-Dalgarno sequences are overlined. Putative -10 promoter elements are underlined. Convergent arrows downstream of *gyrA* indicate the inverted repeat structures possibly functioning as transcription terminators.

*smegmatis* *gyrA*- and *gyrB*-containing plasmids. The ability of *M. smegmatis* gyrase genes to suppress the *gyrA* or *gyrB* temperature-sensitive phenotype of *E. coli* was tested by incubating the transformants spread on LB agar plates at the non-permissive temperature. Plasmids pMN6A and pMN1Bg did not complement. Plasmid pMK99A containing *M. smegmatis* *gyrA* could complement the temperature-sensitive phenotype of *E. coli* NH647. The *gyrB* strain, *E. coli* N4177 harbouring pMK99B (containing the *gyrB* gene) failed to grow at the non-permissive temperature. However, both of the strains could be rescued at 42°C with *E. coli* gyrase plasmids pMK90 and pMK47.

Expression of the *gyrB* and *gyrA* genes of *M. smegmatis* in *E. coli*

In order to overexpress the enzyme, the genes were subcloned into bacteriophage T7 promoter-based expression vectors. The details of the genetic manipulations are described in Methods and presented in Fig. 4. To overcome PCR-induced mutations, only a short fragment between the initiation codon and the first *BglI* site of *gyrB* was amplified and the sequence was confirmed. This novel PCR-based strategy also replaced the initiation codon GTG with ATG, creating an *NcoI* site. The plasmid pMK25 contains the *gyrB* gene cloned into the expression vector pET20b+.

Plasmid pET11d was used to obtain plasmid pMK26, *gyrA*-overexpressing clone. Upon induction of *E. coli* BL21 (DE3)/pLysS cells harbouring the plasmid pMK25, a 75 kDa protein was overproduced (Fig. 5, lane 7). A similar analysis by SDS-PAGE of cell lysates from plasmid pMK26-harbouring cells showed overproduction of a 95 kDa protein (Fig. 5, lane 5). The size of the overproduced proteins is in agreement with the molecular masses calculated from the deduced GyrB and GyrA polypeptides. The level of overproduction is about 25% (GyrB) and 15% (GyrA) of the total protein.

Reconstitution of DNA gyrase activity

Energy-dependent introduction of negative supercoils into covalently closed circular DNA is a unique property of DNA gyrase amongst all the topoisomerases. In order to confirm that the expressed proteins are indeed the subunits of DNA gyrase, the cell extracts from the overexpressing clones fractionated with ammonium

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Fig. 3. Dot matrix comparisons of *gyrB* and *gyrA* of *M. smegmatis*, *M. tuberculosis* and *E. coli*. Amino acid comparisons were made with a window of 30 amino acids at a stringency of 20. The ORFs of the genes were compared with a window of 50 bases at a stringency of 35, starting from the first nucleotide of the ORF. The *E. coli* gene sequences were taken from Swanberg & Wang (1987) for *gyrA* and Adachi et al. (1987) for *gyrB*. Nucleotide and amino acid sequences of gyrase genes of *M. tuberculosis* were from Madhusudan et al. (1994a, b) and Takiff et al. (1994). Comparisons of *gyrB* (a) and *gyrA* (b) amino acid and nucleotide sequences. The short lines and dots parallel to the main diagonal in the GyrA Harr plot indicate the presence of short repeats.

sulfate were assayed for the DNA supercoiling activity and the results are presented in Fig. 6. No DNA supercoiling activity was detected when individual subunit preparations were assayed. However, a strong supercoiling activity could be obtained when the two fractions were combined, resulting in reconstitution of the functional enzyme (Fig. 6, lanes 4 and 5). As little as 100 ng reconstituted protein could convert 50% of the substrate into supercoiled DNA, suggesting a high proportion of active subunits in the preparation. This activity was found to be ATP-dependent. Coumarins such as novobiocin are known to interfere with ATPase activity of subunit B, thereby inhibiting the energy-dependent negative supercoiling of DNA by the enzyme (Reece & Maxwell, 1991a). This antibiotic inhibited the supercoiling activity seen in the combined extract even at the very low concentration of 0.2 μg ml⁻¹ (Fig. 6, lane 7).

**DISCUSSION**

We have determined the complete nucleotide sequence of the structural genes for DNA gyrase from *M. smegmatis*. The comparison of the derived polypeptide sequence with DNA gyrase from other bacteria confirms that the sequence is indeed that of *gyrA* and *gyrB*. The authenticity of the genes was further established by complementation analysis and reconstitution of DNA gyrase activity from the overexpressed subunits. DNA gyrase genes from Gram-positive bacteria and *Mycoplasma* spp. are linked, with *gyrB* preceding *gyrA*. In contrast, the organization of gyrase genes is different in *E. coli*: *gyrB* is located at 83.5 min near *oriC* on the chromosome map, while *gyrA* is at 48 min. The organization of gyrase genes in *M. smegmatis* is similar to that of *M. tuberculosis* (Madhusudan et al., 1994a, b) and Takiff et al. (1994), *B. subtilis* (Moriya et al., 1985) and other Gram-positive bacteria.

In *B. subtilis*, though the genes are contiguous, they are reported to be transcribed independently (Ogasawara et al., 1985). The 211 bp intergenic region has necessary transcription signals. However, the intergenic region of DNA gyrase genes is short (29 bp) in *M. smegmatis* and has no promoter- or terminator-like sequences. Sequences resembling consensus promoters are seen only upstream of the *gyrB* ORF. Large stem–loop structures with the potential to function as transcription terminators are observed downstream of *gyrA*. These inverted repeats are not followed by a 'T-stretch', characteristic of *E. coli* terminators. The actinophage δC31 which infects *Streptomycetes* spp. has been shown to have rho-independent terminators without a 3' poly-U tail (Ingham et al., 1995). A similar organization is found in *M. tuberculosis*, where *gyrB* and *gyrA* are separated by 34 bp. Thus, the DNA gyrase genes of these two species of *Mycobacterium* seem to be transcribed as a single message. It is noteworthy that the initiation codon for *gyrB* is GTG, while that for *gyrA*
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**Fig. 4.** Construction of expression plasmids. (a) GyrB expression strategy: two step cloning procedure involving PCR is depicted. The short arrows indicate the primers used in PCR with the forward primer carrying an Ncol site. (b) Subcloning of gyrA into pET11d. The start and stop codons and the relevent restriction sites are shown.
is ATG. Although GTG functions as an initiation codon, it is known to be less efficient when compared to ATG in *E. coli* (Gold, 1988). The reason for the contiguous arrangement of gyrase genes and the selection of different initiation codons may be to ensure that near-stochiometric amounts of the subunits are synthesized. This could be a means to obviate identical mechanisms operating at two different loci to regulate the expression of gyrase genes, unlike in *E. coli*.

Apart from identical organization, the genes from the two species of *Mycobacterium* are highly homologous. The sequence similarity varies from 60 to 94% with its counterparts from other bacteria. For example, the amino acid sequence of Gyrb shows > 65% similarity with subunit B from other bacteria while the identity is > 50%; with Gyra, the similarity is > 67% but the identity is only ~ 45% compared with other Gyra proteins. However, the regions important for the activity such as the ATPase domain, DNA breakage–reunion domain, etc., are conserved. The gyrb genes of *M. smegmatis* and *M. tuberculosis* show near identity while glyA shows a region of dissimilarity only towards the carboxy-terminus with a stretch of ~ 30 amino acids sharing no homology. However, dot matrix analysis of gyrb genes revealed a difference between the *E. coli* and *M. smegmatis* sequences towards the carboxy-terminus. We have noted the absence of the same stretch of amino acids in Gyrb of *M. tuberculosis* (Madhusudan et al., 1994a).

The overexpressed DNA gyrase subunits of *M. smegmatis* when combined could reconstitute the functional enzyme *in vitro* that catalysed the negative supercoiling of covalently closed circular DNA. No such activity was seen when either of the subunit preparations were used or when ATP was omitted from the reaction. DNA gyrase is a target for well-known compounds such as coumarins and quinolones. Coumarins such as novobiocin and coumermycin A1 inhibit the ATP-dependent supercoiling activity of the enzyme. In this report, we show that low concentrations of novobiocin inhibit the supercoiling activity catalysed by DNA gyrase of *M. smegmatis*. This ATP-dependent, novobiocin-sensitive supercoiling of DNA is characteristic of DNA gyrase.

The near identity of *M. smegmatis* and *M. tuberculosis* genes would facilitate systematic genetic experiments using fast-growing, non-pathogenic *M. smegmatis* strains. The overproduced proteins could be used for studies on structure–function and reaction mechanics. In addition, the proteins could be employed in the assay systems to screen a variety of compounds to identify new potent antimycobacterials. Further experiments with the clones and the enzyme would form a basis to address these important points.

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