

## RESEARCH ARTICLES

# Structural heterogeneity in DNA gyrases from Gram-positive and Gram-negative bacteria

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**GyraseA (GyrA) subunit of DNA gyrase from mycobacteria has certain characteristics distinct from that of *E. coli*. Polyclonal antibodies produced against *M. tuberculosis* GyrA recognized GyrA from different slow and fast growing mycobacterial species and also from several Gram-positive bacteria. However, these antibodies did not cross-react with *E. coli* GyrA and the enzyme from other Gram-negative bacteria. The results from the present study together with multiple alignment, pairwise comparison and biochemical properties support the idea of the occurrence of two subclasses of gyrases in the bacterial kingdom, emphasizing the importance of the enzyme as a molecular target for the development of novel therapeutics.**

DNA gyrase is unique amongst all known topoisomerases, as it is the only enzyme capable of introducing negative supercoils into DNA<sup>1-3</sup>. The enzyme is found only in bacteria and is essential for cell survival, since negative supercoiling of DNA is a prerequisite during replication, transcription and other DNA transaction processes<sup>2</sup>. DNA gyrase has been extensively studied from *E. coli* with respect to structure–function, biochemistry, oligomeric status and mechanistic of the supercoiling reaction<sup>4-10</sup>. The *E. coli* enzyme is a heterotetramer, and consists of two subunits each of gyraseA (GyrA) and gyraseB (GyrB) encoded by *gyrA* and *gyrB* genes, respectively<sup>5</sup>. GyrA is thought to be principally concerned with the breakage and reunion of DNA<sup>6</sup>, while GyrB subunit is the site of ATP hydrolysis<sup>11</sup>. The enzyme is the target of two major groups of antibacterial agents, the quinolones and coumarins. Since gyrase is a proven drug target, there has been considerable interest to study gyrase from different bacteria. As a consequence, gyrase genes have been characterized from *Klebsiella pneumoniae*<sup>12</sup>, *Pseudomonas aerogenosa*<sup>13</sup>, *Mycoplasma pneumoniae*<sup>14</sup>, *Staphylococcus aureus*<sup>15</sup>, *Bacillus subtilis*<sup>16</sup>, *Streptomyces coelicolor*<sup>17</sup>, *Mycobacterium tuberculosis*<sup>18,19</sup>, *M. smegmatis*<sup>20</sup>, *M. leprae*<sup>21</sup> and many others. In most of these cases, studies have been confined to elucidating the mechanism of drug resistance, by identifying mutations in the gyrase genes.

The genus mycobacterium comprises more than fifty species and about twenty of them are of medical importance. Besides the most important mycobacterial pathogens such as *M. tuberculosis* and *M. leprae*, the respective causative agents of tuberculosis and leprosy, other mycobacteria such as *M. avium*, *M. kansasii*, *M. chelonae* and *M. fortuitum* can cause opportunistic infections in immunocompromised hosts. In order to address the role of gyrase in important processes in mycobacteria, for biochemical characterization and to develop it as a suitable drug target, we have cloned *gyrB* and *gyrA* from *M. smegmatis*<sup>20</sup> and *M. tuberculosis*<sup>18</sup>. These studies are important considering the worldwide reemergence of mycobacterial infections and the evolution of multidrug resistant (MDR) strains of *M. tuberculosis*<sup>22</sup>.

In this article, we describe the over-expression of GyrA from *M. tuberculosis*, generation of polyclonal antibodies and their utility in assessing the cross-reactivity of GyrA subunits from various bacteria. The lack of immunological cross-reactivity between Gram-positive and Gram-negative bacteria, along with sequence analysis of gyrase genes and other observations form the basis for us to suggest the existence of two subclasses of DNA gyrases.

## Materials and methods

### *Bacterial strains, plasmids and materials*

*M. smegmatis* SN2 (Msg) and *E. coli* DH10b (Eco) were laboratory strains. *M. tuberculosis* H37Rv (Mtb) was obtained from National Tuberculosis Institute, Bangalore, India. *M. avium*, *M. bovis*, *M. kansasii*, *M. fortuitum*, *M. gilvum*, *M. phlei* and *M. voccae* were provided by Dr V. M. Katoch, Central JALMA Institute for Leprosy, Agra, India. *M. leprae* (Mlp) cell lysate was a kind gift from Dr P. J. Brennan, Colorado State University, Colorado, USA. Other Gram-positive bacteria, viz. *S. coelicolor* (Sco), *B. subtilis* (Bsu) and *S. aureus* (Sau) and Gram-negative bacteria, viz. *P. aerogenosa* (Pae), *K. pneumoniae* (Kpn), and *Salmonella typhimurium* were obtained from the Institute of Microbial Technology, Chandigarh, India. Different mycobacteria were grown in modified

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Youmans and Karlson's medium<sup>23</sup> with 0.2% Tween-80 at 37°C. All other bacteria were grown according to the provider's specifications. Restriction endonucleases and modifying enzymes were from Boehringer Mannheim, Amersham Pharmacia Biotech and New England Biolabs and used according to manufacturers' specifications. All other reagents were purchased from Sigma. pET20b+ plasmid was obtained from Novagen.

#### Preparation of cell-free extracts from bacterial strains

The cells were harvested at mid log phase by centrifugation at 6000 rpm for 20 min at 4°C and resuspended in sonication buffer (20 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol, 5 mM *b*-mercaptoethanol, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 1 mM benzamidine HCl and 1% Triton X-100). The cells were lysed by sonication (Virtis 475 Sonicator) and cell-free extracts were collected by centrifugation at 12,000 rpm for 30 min at 4°C. Protein was estimated by the method of Bradford<sup>24</sup>.

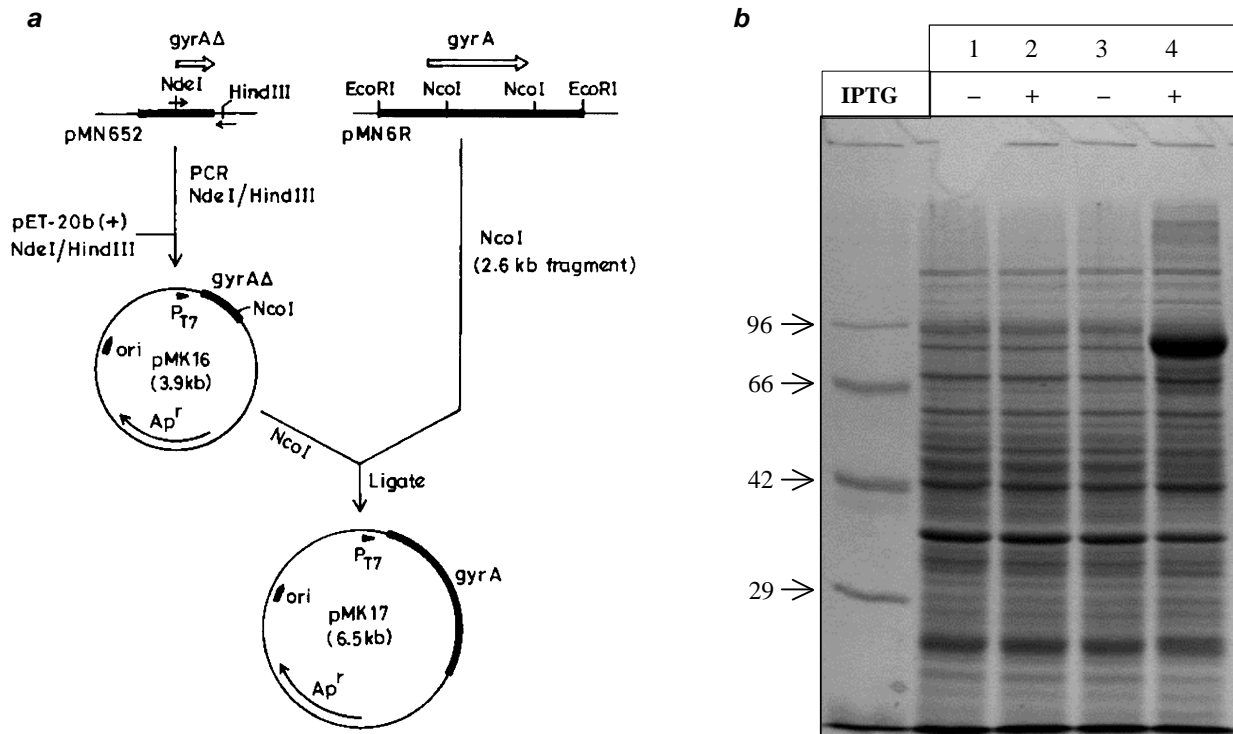
#### Hyper expression of GyrA and generation of polyclonal antibodies

A 257 bp fragment from the 5' end of *gyrA* ORF was amplified by PCR from the genomic clone pMN652 (ref. 18)

using a 5' primer with a *NdeI* site providing the ATG start codon (5' TGAATTCATATGACTGATACGACG 3') and the pTZ19U reverse primer (5' CAGGAAACAGCTATGAC 3') as the 3' primer. The PCR product was digested with *NdeI* and *HindIII* and cloned into *NdeI/HindIII* digested pET20b+ to obtain pMK16. The cloned DNA fragment was sequenced to confirm the absence of any mutations. The remaining part of the *gyrA* gene, a 2.6 kb *NcoI* fragment from a genomic clone pMN6R<sup>18</sup>, was cloned at the *NcoI* site in pMK16 (Figure 1 a) to obtain pMK17.

pMK17 was transformed into the *E. coli* strain BL21 (DE3) pLysS and cells induced with IPTG (300 µM) for three hours. Cells were collected by centrifugation, resuspended in sonication buffer and lysed by sonication. The cell lysate was centrifuged at 12,000 rpm to obtain the pellet which contained the GyrA protein in inclusion bodies. The pellet was washed thrice in 10 mM phosphate buffer (pH 7.2) with 0.8% NaCl, and 0.1% Triton X-100 and subsequently three more times with the same buffer without detergent. The protein band corresponding to GyrA was purified by electroelution. Recombinant *E. coli* GyrA was purified from DH10b cells harbouring pPH3 plasmid as described by Maxwell and Howells<sup>25</sup>.

Antibodies were raised against purified Mtb GyrA protein in rabbits as described<sup>26</sup>. The antibody titer was determined using Enzyme Linked Immunosorbent



**Figure 1.** a, Cloning strategy for *M. tuberculosis gyrA* gene in pET-20b+. The two-step cloning procedure involving PCR is depicted. Short arrows indicate the primers used in PCR with the forward primer carrying an *NdeI* site. The relevant restriction sites are shown; b, SDS-polyacrylamide gel electrophoresis of *E. coli* BL21 (DE3)/pLysS cell lysates harbouring pET-20b+ without induction (lane 1) or induction with IPTG (lane 2). *E. coli* cells harbouring plasmid pMK17 uninduced (lane 3) and induced with IPTG (lane 4). The size of the protein molecular weight markers is indicated.

Assay (ELISA) by coating 200 ng of MtbGyrA protein as described earlier<sup>26</sup>.

### Immunoblotting techniques

SDS-PAGE was performed using 8% polyacrylamide separating gels<sup>27</sup>. Proteins from the gel were transferred to PVDF membrane and probed with respective antibodies as described earlier<sup>26</sup>. Bound antibodies were detected by enhanced chemiluminescence (Amersham ECL Plus) according to the manufacturer's instructions.

### Sequence analysis

UWCGC software was used for sequence analysis at the Distributed Information Centre, Indian Institute of Science, Bangalore, India. The pairwise amino acid sequence comparison was performed using GAP and the phylogenetic tree was constructed using PILE UP. MACAW program<sup>28</sup> was used for the multiple alignment of different GyrA proteins.

## Results and discussion

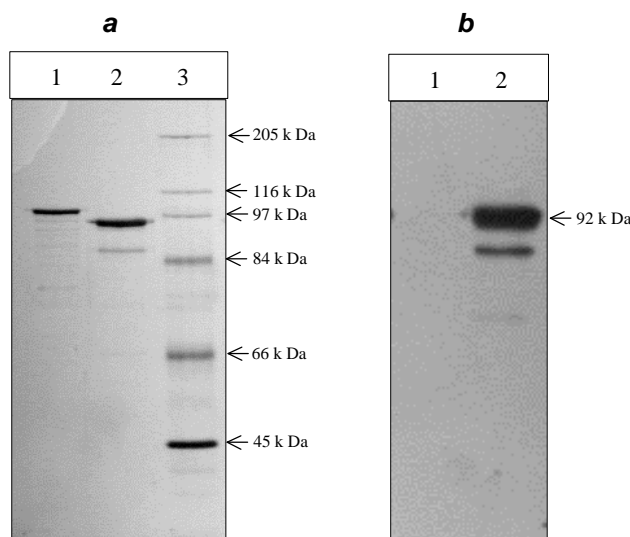
The cloning strategy for GyrA is depicted in Figure 1 *a*. The strategy involved PCR amplification and cloning of the amino terminal small region and then direct in-frame insertion of a genomic fragment containing the rest of the gene. The full-length reconstructed gene is under the control of the  $\Phi 10$  promoter of T7 bacteriophage in pET20b+. *E. coli* BL21 (DE3) pLysS cells harbouring pMK17 plasmid when induced with IPTG, expressed large amounts of protein (Figure 1 *b*), and the size of the over-expressed protein (92.5 kDa) corresponded to the expected molecular mass deduced from the primary sequence. Most of the over-expressed protein was localized to inclusion bodies but this facilitated its purification by electroelution.

In order to assess the immunological relatedness between GyrA from different bacteria, polyclonal antibodies were raised against mycobacterial GyrA subunit. The antibodies generated were of high titer, based on quantitative ELISA using *M. tuberculosis* GyrA. Cross-reactivity of GyrA polyclonal antibodies with *E. coli* GyrA was evaluated and for this purpose *E. coli* GyrA was over-expressed and purified as described<sup>25</sup>. *M. tuberculosis* GyrA is 6 kDa smaller than the 98 kDa *E. coli* GyrA (Figure 2 *a*). In spite of 46.6% identity (59.8% similarity) at the amino acid level between *E. coli* and *M. tuberculosis* GyrA subunits, the antibodies did not show cross-reactivity with the *E. coli* enzyme (Figure 2 *b*, lane 1). Similar results were obtained even at a lower dilution of the antiserum. The polyclonal antibodies raised in mouse against *M. smegmatis* GyrA also did not

cross-react with *E. coli* protein suggesting that it is not a limitation of the experimental protocol (data not shown). We have extended these studies further by employing two different monoclonal antibodies generated against *E. coli* GyrA subunit recognizing different regions on *E. coli* GyrA<sup>29</sup>. These mAbs also did not cross-react with *M. tuberculosis* GyrA. It should be noted that GyrA from *E. coli* and *M. tuberculosis* share several conserved motifs. The absence of cross-reactivity between GyrA from two different species of bacteria though surprising, has also been reported earlier. *E. coli* GyrA polyclonal antibodies did not cross-react with the GyrA subunit of *B. subtilis*<sup>30</sup>.

The cross-reactivity of antibodies raised against Mtb GyrA with other Gram-positive and Gram-negative bacteria was tested. Significant cross-reactivity of Mtb GyrA antiserum with cell extracts from Gram-positive bacteria was observed (Figure 3 *a*). The size of the immunoreactive protein in these cell extracts corresponded to the expected molecular mass of GyrA, except in the case of *S. aureus*. The abnormal faster mobility in the latter case could be due to rapid degradation of the protein, possibly an intrinsic feature of that polypeptide. In contrast to the results with Gram-positive bacteria, hardly any cross-reactivity with *M. tuberculosis* GyrA immune serum was observed with cell-free extracts from Gram-negative bacteria (Figure 3 *b*).

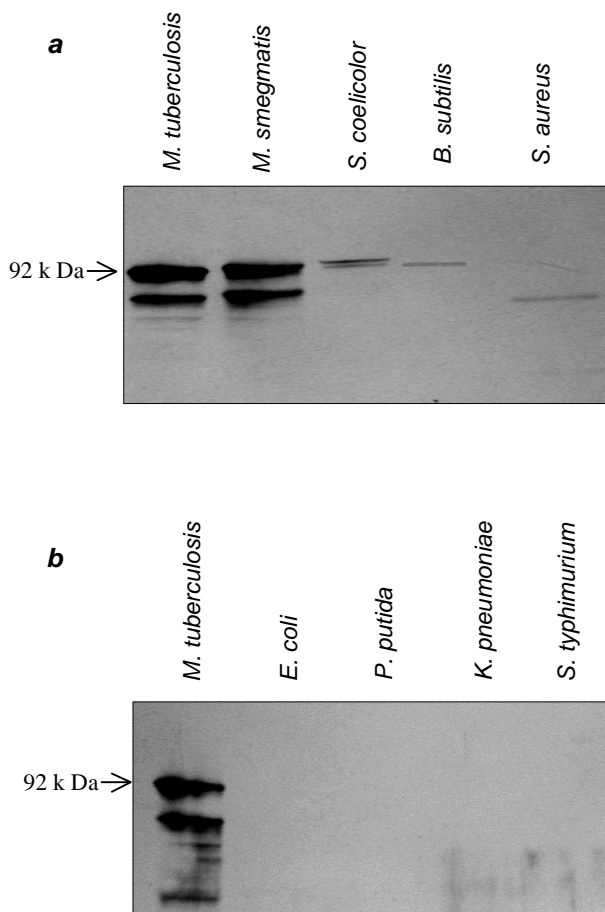
The cross-reactivity profile of anti Mtb GyrA antibodies with various bacterial species indicates that the antigenicity of GyrA is uniquely conserved in Gram-positive bacteria. These results correlate well with the extent of similarity shared by different GyrA polypeptides (Table 1), which show a higher degree of sequence



**Figure 2.** Specificity of Mtb :GyrA polyclonal antibody. SDS-polyacrylamide gel electrophoresis (*a*) and Western blot analysis (*b*) of 1  $\mu$ g each of purified *E. coli* GyrA (lane 1) and *M. tuberculosis* GyrA (lane 2). Antiserum to MtbGyrA was used at a 1 : 10000 dilution. Sizes of molecular mass markers (lane 3) are indicated on the right.

similarity amongst Gram-positive bacteria than Gram-negative bacteria. The immunological difference between different GyrA subunits also seems to correlate well with their pattern in the unrooted phylogenetic tree (Figure 4).

The polyclonal antiserum was tested for cross-reactivity with different mycobacterial species belonging to fast and slow growing groups and the results are presented in Figure 5 *a* and *b*, respectively. Mtb GyA antiserum cross-reacted well with both fast growing and slow growing mycobacteria, indicating that GyrA is very similar among different mycobacterial species with respect to size and immunogenicity of the protein. The antibodies also cross-reacted with *M. leprae* GyrA. It should be noted that in *M. leprae*, the size of the mature GyrA polypeptide is 853 amino acids which is initially synthesized as a precursor containing a 420 amino acid intein<sup>21</sup>. The intein-containing precursor could not be detected by Western blot analysis, indicating the rapid processing of precursor protein to form the mature 95 kDa form.

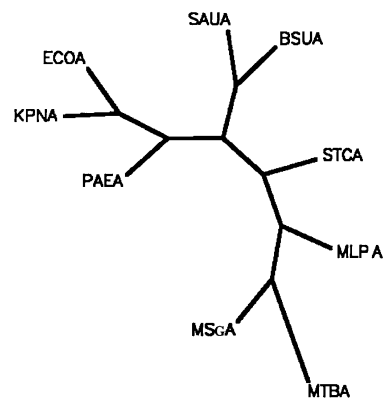


**Figure 3.** Immunological cross-reactivity of DNA gyrase from various Gram-positive (*a*) and Gram-negative (*b*) bacteria. 30  $\mu$ g of protein from different bacterial cell lysates was subjected to 8% SDS-PAGE and blotted onto PVDF membranes. The blots were probed with 1 : 10000 (*a*) and 1 : 5000 (*b*) dilution of anti-Mtb GyrA antiserum and processed as described in the materials and methods section.

An alignment of the amino acid sequences of the GyrA subunits is shown in Figure 6. The N-terminus of GyrA from different Gram-positive and Gram-negative bacteria is largely conserved. In *E. coli* GyrA, tyrosine-122 is responsible for transient DNA scission via a DNA-phosphotyrosine intermediate<sup>31</sup> and in the various protein sequences surrounding the active site, tyrosine (AAMRYTE) is highly conserved. In contrast, the carboxy terminal domain (CTD) of GyrA proteins is divergent. The differences in CTD, a region which is responsible for DNA binding, could imply subtle differences in DNA-binding properties of the enzyme. Furthermore, a stretch of 34 amino acids, located towards the middle of the protein in Gram-negative bacteria is absent in all GyrAs from Gram-positive bacteria and the importance of this region is yet to be elucidated.

A similar comparison of GyrB proteins in various bacteria also shows regions of similarity towards the amino terminal. Two important residues, Glu-42 and His-38, implicated in ATPase activity of GyrB of *E. coli*<sup>32</sup>, are highly conserved in all the GyrB sequences. We have earlier noted a major difference in the primary amino acid sequences of GyrB of Gram-positive and Gram-negative bacteria<sup>33</sup>. A contiguous stretch of 165 amino acids is missing from Gram-positive bacteria and mycoplasma, which is present in *E. coli* GyrB and other Gram-negative bacteria. Our studies have indicated that this region is essential for the DNA-binding ability of the active gyrase tetramer, and deletion of this stretch impairs activity of *E. coli* gyrase both *in vivo* and *in vitro*<sup>34</sup>. These results suggest the presence of an alternate DNA binding region in GyrB of Gram-positive bacteria.

The differences in the properties of DNA gyrase from two major eubacterial groups are further apparent on the basis of studies using the quinolone class of drugs. Data from several laboratories indicate that quinolones are targetted primarily to DNA gyrase in Gram-negative bacteria; topoisomerase IV serves as a secondary target<sup>35,36</sup>. In contrast, topoisomerase IV appears to be the primary

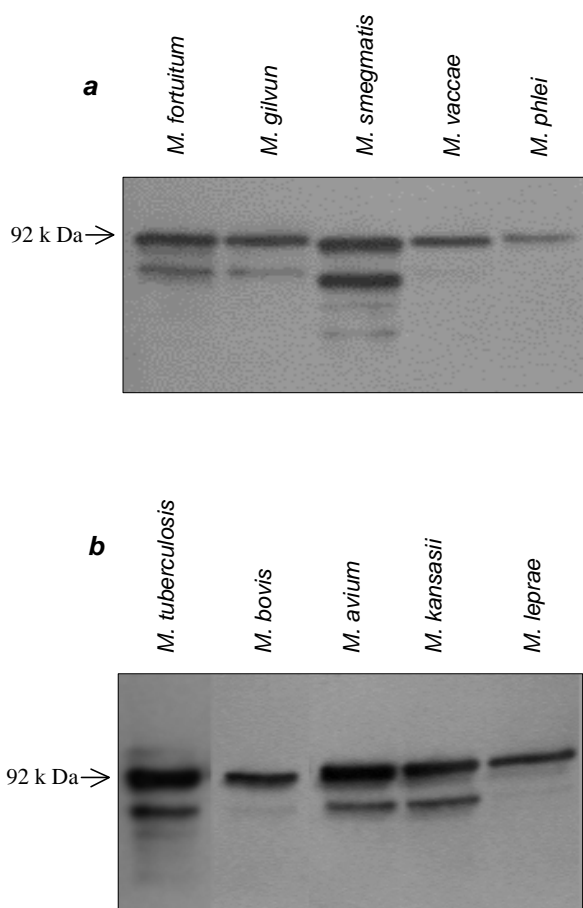


**Figure 4.** Evolutionary relationship among GyrA subunits. Unrooted phylogenetic trees produced from the alignment of GyrA subunits using Phylip.

**Table 1.** Pairwise comparison of GyrA subunits (% similarity/% identity)\* from different bacteria

	Mtb	Mlp	Msg	Sco	Bsu	Sau	Pae	Kpn	Eco
Mtb	100 100	92.7 90.1	93.7 89.7	75.4 65.5	60.6 49.5	59.4 47.6	58.7 46.5	59.7 46.9	59.8 46.6
Mlp	92.7 90.1	100 100	91.2 86.9	72.9 63.5	60.8 49.8	58.4 46.4	58.8 45.1	58.7 45.8	58.8 46.2
Msg	93.7 89.7	91.2 86.9	100 100	75.4 66.1	61.1 50.2	58.3 47.4	58.4 46.1	60.9 47.2	60.4 47.1
Sco	75.4 65.5	72.9 63.5	75.4 66.1	100 100	60.4 49.0	59.6 48.7	58.9 47.3	59.6 48.2	59.6 47.7
Bsu	60.6 49.5	60.8 49.8	61.1 50.2	60.4 49.0	100 100	74.9 64.7	65.5 53.4	65.6 53.7	66.2 53.9
Sau	59.4 47.6	58.4 46.4	58.3 47.4	59.6 48.7	74.9 64.7	100 100	61.8 48.6	63.2 50.8	64.3 51.6
Pae	58.7 46.5	56.8 45.1	58.4 46.1	58.9 47.3	65.5 53.4	61.8 48.6	100 100	76.7 66.5	77.7 67.1
Kpn	59.7 46.9	58.7 45.8	60.9 47.2	59.6 48.2	65.6 53.7	63.2 50.8	76.7 66.5	100 100	92.6 89.4
Eco	59.8 46.6	58.8 46.2	60.4 47.1	59.6 47.7	66.2 53.9	64.3 51.6	77.7 67.1	92.6 89.4	100 100

\*In each cell, the top value is % similarity and the bottom value is % identity.

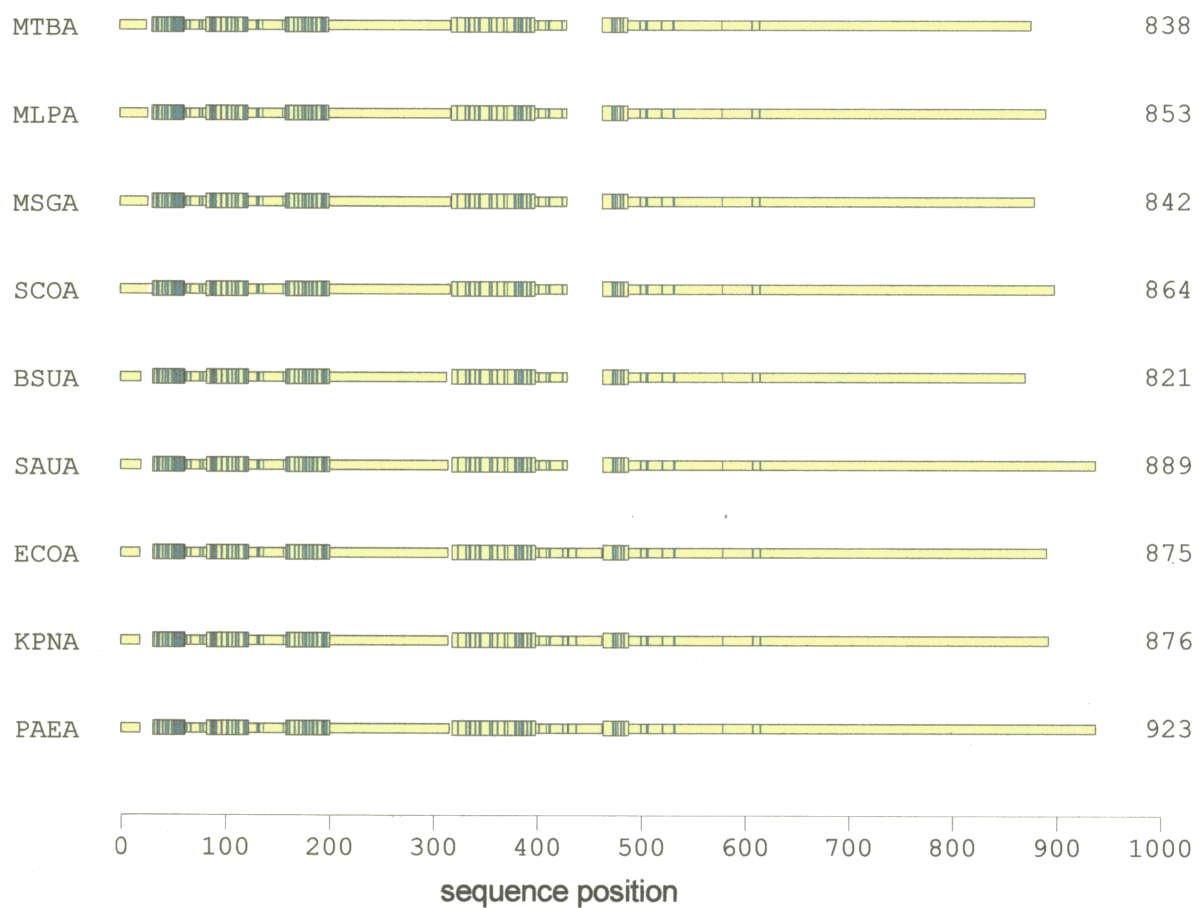


**Figure 5.** Immunological characterization of DNA Gyrase from different mycobacteria species by Western blot analysis. 20  $\mu$ g of fast growing (a) and slow growing (b) mycobacterial cell lysates was subjected to SDS-PAGE and further analysed by immunoblotting with 1 : 20000 Mtb anti-GyrA antiserum.

cytotoxic target for most members of this drug family in Gram-positive bacteria<sup>37</sup>.

Despite the highly conserved region near oriC in the genome of eubacteria, there is a difference in *gyrB* and *gyrA* gene arrangements in Gram-positive and Gram-negative bacteria. In all the characterized Gram-positive bacteria, i.e. *S. aureus*<sup>15</sup>, *B. subtilis*<sup>16</sup>, *S. sphaeroides*<sup>38</sup>, *M. tuberculosis*<sup>18</sup>, *M. smegmatis*<sup>20</sup>, *M. leprae*<sup>39</sup> and halophilic archaeobacteria<sup>40</sup>, *gyrA* and *gyrB* genes are contiguous or found in close proximity to each other. In most of the Gram-negative bacteria, i.e. *E. coli*<sup>41</sup>, *P. aerogenosa*<sup>42</sup> and *K. pneumoniae*<sup>12</sup> however, the genes are found far apart on the chromosome. We have shown earlier that gene structure, transcriptional organization and regulation of the *gyr* operon in mycobacteria are different from that of *E. coli*<sup>43</sup>. From these results, it appears that in spite of substantial similarities at the amino acid level, there are significant differences in the structure and regulation of gyrase enzyme in different bacteria.

Taken together, these results lead us to propose the existence of two subclasses of gyrases represented in Gram-positive and Gram-negative bacteria. It is clear that the overall reaction cycle catalysed by the members of the either class is similar, as motifs important for catalysis are conserved in all the gyrase sequences characterized so far. The differences observed in the gene structure and protein sequence could have evolved in a species or genera-specific manner. These differences could be involved in subtly influencing catalytic properties and the modulation of enzyme function. A detailed comparative study of the biochemical properties of gyrase from these two different classes would be necessary to understand the functional basis for the differences in order to exploit



**Figure 6.** Schematic representation of a multiple alignment of GyrA subunits using MACAW program<sup>28</sup>. The thick blocks correspond to sequences sharing significant similarity and the shaded regions represent the conserved sequence. The thin blocks represent the regions of the sequence not sharing statistically significant homology in the multiple alignment.

the enzyme as a specific molecular target for the development of new drugs.

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