An orphan *gyrB* in the *Mycobacterium smegmatis* genome uncovered by comparative genomics

P. JAIN and V. NAGARAJA*

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India

Abstract

DNA gyrase is an essential topoisomerase found in all bacteria. It is encoded by *gyrB* and *gyrA* genes. These genes are organized differently in different bacteria. Direct comparison of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* genomes reveals presence of an additional *gyrB* in *M. smegmatis* flanked by novel genes. Analysis of the amino acid sequence of GyrB from different organisms suggests that the orphan GyrB in *M. smegmatis* may have an important cellular role.

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Introduction

Topoisomerases are the enzymes that interconvert between different topological forms of DNA. On the basis of structure and mechanism of reaction, the enzymes are broadly classified into type I and type II topoisomerases (Liu *et al*. 1980; Berger *et al*. 1998). Type I topoisomerases cleave only one strand of DNA and change the linking number in steps of one. Type II topoisomerases on the other hand cleave both strands of DNA to form a 5′-phosphotyrosine linkage, pass another duplex segment of DNA through the break, and religate the broken ends (Wang 1996). Thus, unlike type I topoisomerases, type II topoisomerases change the linking number in steps of two (Brown and Cozzarelli 1979; Liu *et al*. 1980). DNA gyrase, topoisomerase IV of bacteria and eukaryotic topoisomerase II are typical members of the latter class. All type II topoisomerases have the ability to catalyse ATP-dependent catenation/decatenation and knotting/ unknotting of DNA (Cozzarelli 1980; Gellert 1981) and show high degree of conservation in their primary sequence (Madhusudan and Nagaraja 1996). Among all the topoisomerases, bacterial DNA gyrase—a type II enzyme—is the only enzyme capable of introducing negative supercoils into DNA (Wang 1996).

DNA gyrase was initially discovered in *E. coli* in 1976 (Gellert *et al*. 1976a). The enzyme from *E. coli* has been subjected to extensive investigation with respect to gene organization and regulation, biochemical characterization, reaction mechanism and interaction with various drugs (Maxwell and Gellert 1986; Reece and Maxwell 1991; Wang 1996; Maxwell 1997; Champoux 2001). However, every bacterial genome analysed contains DNA gyrase. The enzyme is a heterotetramer, and is encoded by two genes, *gyrB* and *gyrA*, which encode important functional domains in the enzyme. DNA breakage and religation activity, which is sensitive to the quinolone class of drugs, resides in GyrA subunit (Gellert *et al*. 1977; Sugino *et al*. 1977). ATPase activity, which is inhibited by the coumarin class of drugs, resides in GyrB, providing essential energetics for the reaction cycle (Gellert *et al*. 1976b). Only the holoenzyme comprising two GyrA and two GyrB subunits is capable of catalysing the overall supercoiling reaction (Klevan and Wang 1980; Sugino *et al*. 1980). The indispensability of DNA gyrase could be demonstrated by the inhibition of cell growth by various inhibitors of the enzyme. This has led to intense study to

*For correspondence. E-mail: vraj@mcbl.iisc.ernet.in.

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develop novel anti-infective agents. As a result, the genes have been cloned from a large number of organisms. We have previously cloned genes for DNA gyrase (*gyrB* and *gyrA*) from *Mycobacterium smegmatis* and *M. tuberculosis* (Madhusudan *et al*. 1994; Madhusudan and Nagaraja 1995) for detailed characterization of the enzyme and to develop them as targets for novel compounds. DNA gyrase from mycobacteria differs from the *E. coli* enzyme with respect to susceptibility to various classes of inhibitors, antibody cross-reactivity, primary structure and biochemical characteristics (Chatterji *et al*. 2000, 2001; Manjunatha *et al*. 2000, 2001a,b, 2002). These studies have led to the classification of DNA gyrase into two subgroups within the prokaryotes (Manjunatha *et al*. 2000).

Four topoisomerases have been characterized from *E. coli*. Topoisomerase I and topoisomerase III are type IA enzymes. DNA gyrase and topoisomerase IV are type II enzymes. So far, no additional copy of any of the genes encoding a topoisomerase has been reported in *E. coli*. Here we report the uncommon presence of an additional *gyrB* in the *M. smegmatis* genome and examine its origin. Further, we describe the heterogeneity in organization of the *gyr* locus in different bacteria.

Materials and methods

M. smegmatis genome sequence was queried using WU-BLAST 2.0 (TIGR server, http://tigrblast.tigr.org/tgi/). The sequence was further queried using BLASTp (NCBI BLAST 2.0; default options). GyrB sequences were retrieved from the EMBL database.

Programs used in the sequence analysis were part of the sequence analysis package from the University of Wisconsin Genetics Computer Group (UWGCG), version 9.1. The following programs were used with the default settings: Gap for pairwise alignment, ClustalW for multiple sequence alignment, Macaw to determine the regions of local similarity and to generate similarity boxes of statistical significance.

Southern blotting was carried out using genomic DNA as described earlier (Madhusudan *et al*. 1994).

Results and discussion

Additional gyrB in M. smegmatis

We have previously cloned and overexpressed *gyrA* and *gyrB* genes from both *M. smegmatis* (Madhusudan and Nagaraja 1995) and *M. tuberculosis* (Madhusudan *et al*. 1994). DNA fragments comprising *gyrB* or *gyrA* or both from *M. tuberculosis* were used to probe for homologous genes in *M. smegmatis* and *M. tuberculosis*. Southern analysis of genomic DNA showed additional DNA fragments hybridizing in case of *M. smegmatis* which cannot be accounted for in the restriction map of *gyrBA* locus (figure 1). In contrast, *M. tuberculosis* DNA blots did not show the additional bands (not shown). These results suggested the presence of additional DNA sequences or genes similar to gyrase genes in *M. smegmatis* which are absent in *M. tuberculosis*. Analysis of genome sequences of the two organisms confirmed these observations. In the entire 4,411,529-bp genome of *M. tuberculosis* only *gyrB*, *gyrA* and *topA* genes are found (Cole *et al*. 1998); the genes encoding topoisomerase IV or topoisomerase III are absent (Cole *et al*. 1998). However, sequence analysis of the *M. smegmatis* genome revealed presence of a gene for an additional GyrB protein, confirming the result of Southern analysis (figure 1). It should be noted that only the *gyrB*-specific probe picked up the additional hybridizing fragments (figure 1), which suggests that the additional *gyrB* is located away from the main *gyrBA* locus in the genome. The GyrB homologue shows a high degree of similarity of amino acid sequence to GyrB from *M. smegmatis* (table 1). No corresponding *gyrA* was found next to this additional *gyrB* sequence unlike in the *gyrBA* operon of *M. smegmatis* and *M. tuberculosis* (Unniraman and Nagaraja 1999; Unniraman *et al*. 2002). Further, no additional *gyrA* was found elsewhere in the genome. We therefore refer to the new *gyrB* homologue as 'orphan' *gyrB* and to the product as orphan GyrB.

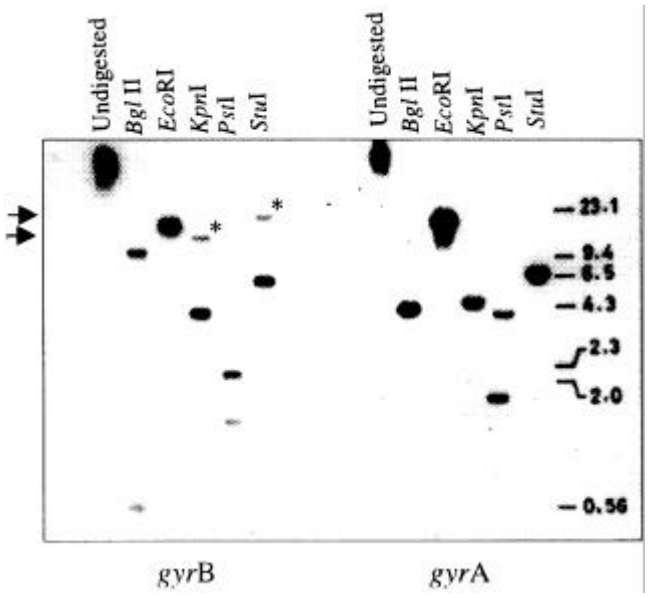

Figure 1. Southern analysis of *M. smegmatis* DNA. Left, Hybridization of *M. smegmatis* SN2 genomic DNA with 1.2-kb *Eco*RI–*Hpa*I fragment of pMN13R carrying *M. tuberculosis gyrB* gene (Madhusudan *et al*. 1994). Right, Hybridization of *M. smegmatis* SN2 genomic DNA with 1.0-kb *Mlu*I–*Sma*I fragment of pMN6R carrying *M. tuberculosis gyrA* gene (Madhusudan *et al*. 1994). Identical length fragments hybridize to both probes in *Eco*RI, *Kpn*I and *Stu*I digests. Additional hybridization signals in *Kpn*I and *Stu*I digests (left panel) are indicated by an asterisk.

Table 1. Amino acid sequence homology among GyrB sequences by pairwise alignment.

	M. tuberculosis GyrB	*M. smegmatis* GyrB	*M. smegmatis* orphan GyrB
M. tuberculosis GyrB	100		
M. smegmatis GyrB	91.9	100	
M. smegmatis orphan GyrB	88.5	89.9	100

Characteristics of orphan GyrB

The orphan GyrB sequence was compared with the already characterized GyrB of *M. smegmatis* and *M. tuberculosis* (table 1). The orphan GyrB shows 85.9% and 76.2% identity, and 89.9% and 88.5% similarity to *M. smegmatis* and *M. tuberculosis* GyrB respectively. Moreover, the region important for ATPase activity is highly conserved between orphan GyrB and canonical GyrB of *M. smegmatis*. Orphan GyrB sequence was then compared with the GyrB sequences of other bacteria. The similarity ranged from 40% to 70%, indicating that orphan *gyrB* is a close allele of authentic *gyrB*. These results imply that orphan *gyrB* is likely to have originated from the authentic *gyrB* of *M. smegmatis* and not transferred from a foreign genome. However, analysis of the primary sequence of orphan GyrB reveals deletion of a 30-amino-acid stretch corresponding to residues 219–248 of *M. smegmatis* GyrB, raising the possibility that the gene is vestigial (figure 2a). Pairwise alignment was carried out to determine presence or absence of this 30-amino-acid stretch in GyrB of various organisms. To represent the regions of local similarity and to generate similarity boxes of statistical significance Macaw alignment was carried out (figure 2b). The additional 30 amino acids found in *M. smegmatis* GyrB are absent in GyrB of many other Gram-positive and Gram-negative bacteria, including *E. coli*, indicating that this region is likely to be dispensable for GyrB activity (figure 2b). However, this stretch is present in the GyrB of all mycobacterial genomes sequenced so far, and deletion of fewer amino acids is seen in *Streptomyces sphaeroides* and *S. coelicolor* GyrB sequences. The presence of an additional 30 amino acids in GyrB of some bacteria raises the possibility that this region may have a role in species-specific function (see later section). The overall similarity of orphan GyrB with authentic GyrB from other bacteria suggests that the orphan gene is a functional allele in *M. smegmatis*.

Organization of gyr locus and location of orphan gyrB

Organization of the *gyr* locus shows considerable diversity in different bacteria. In *E. coli gyrB* is located at 83 min on the circular genome, close to the origin of replication, *oriC* (Bachmann and Low 1980). Although in most organisms *gyrB* is found in close proximity to the origin, the location of *gyrA* varies from organism to organism. *E. coli gyrA* is located far away from the origin at 48 min (Bachmann and Low 1980). In contrast to all the other known systems, in *M. smegmatis* and *M. tuberculosis gyrB* and *gyrA* are organized in the form of an operon (Unniraman and Nagaraja 1999; Unniraman *et al*. 2002). The presence of an orphan *gyrB* in *M. smegmatis* prompted us to look for its location in the genome and organization of *gyrB* and *gyrA* genes in different mycobacterial species (figure 3). It is possible that the orphan *gyrB* and the flanking regions arose by partial genome duplication events, as is the case in *M. bovis* BCG Pasteur strain (figure 3, and Brosch *et al*. 2000). Alternatively, only the *gyrB* sequence could have been inserted, either by transposition or by recombination events. The gene organization around *oriC* and the *gyr* locus in *M. bovis*, *M. tuberculosis* and *M. smegmatis* is nearly identical (figure 3). The duplication event seen in *M. bovis* BCG Pasteur strain does not appear to be a common feature (figure 3). The orphan *gyrB* in *M. smegmatis* is present at a distinct locus flanked by unknown open reading frames. Nucleotide sequences flanking the orphan *gyrB* do not provide any clues regarding its insertion, which suggests it may have been an early event.

The presence of this additional *gyrB* with the characteristics of a functional allele raises many interesting questions regarding its physiological role. Although an additional *gyrB* is uncommon in bacteria, there is a precedent in *S. sphaeroides*. The indispensability of DNA gyrase for cell survival is exploited in nature by competing organisms by evolution of inhibitors and poisons. Such organisms develop and retain their own defensive strategies. *S. sphaeroides* is the natural producer of the antibiotic novobiocin, which is a potent inhibitor of DNA gyrase of all bacteria. This organism protects itself from the action of novobiocin by synthesizing both sensitive and resistant GyrB proteins, encoded by two genes, $gyrB^S$ and $gyrB^R$. There is only one *gyrA* gene in the genome, present downstream of $gyrB^S$ and cotranscribed with it (Thiara and Cundliffe 1993). Production of $GyrB^R$ along with GyrA permits survival of *S. sphaeroides* in presence of novobiocin over other species which do not have a novobiocin-resistant GyrB. It has been shown that certain drugs like cisplatin also inhibit *E. coli* DNA gyrase activity and preferentially induce *gyrB* gene expression (Neumann *et al*. 1996). Other inhibitors of gyrase might exist, which bind to the GyrB subunit to inhibit GyrA–

(a)

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Orphan GyrB  IARRLQEMAFLNKGLILTLTDQR---------------DPEPQT-----------------R
GyrB         VARRLQEMAFLNKGLTIELTDERVTAEEVVDDVVKDTAEAPKTADEKAAEATGPSKVKHR

Orphan GyrB  TFHHPGGLIDYVKHINRVKDPIQPSIIAFEGEGPGHEVEIAMQWNAGYSESVHTFANTIN
GyrB         VFHYPGGLVDYVKHINRTKTPIQQSIIDFDGKGPGHEVEIAMQWNAGYSESVHTFANTIN
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(b)

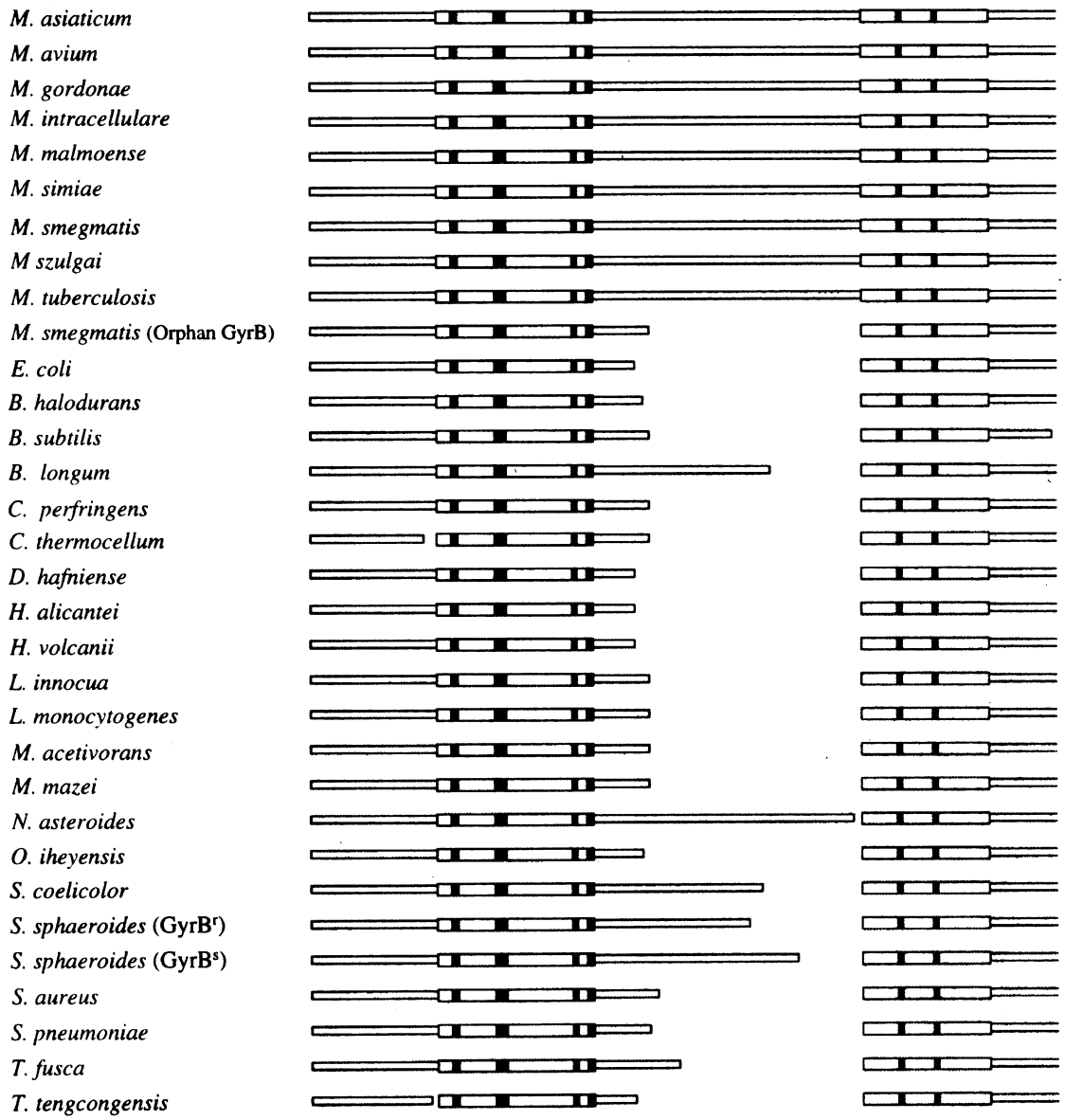

Figure 2. Comparison of GyrB sequences. (a) Pairwise alignment of the intragenic region between authentic GyrB and orphan GyrB of *M. smegmatis*. Amino acid residues from 180 to 290 are aligned. (b) Schematic representation of multiple sequence alignment. The program Macaw was used to represent the statistically significant homology in multiple sequence alignment. The sequences aligned are from the region of GyrB shown in *a*.

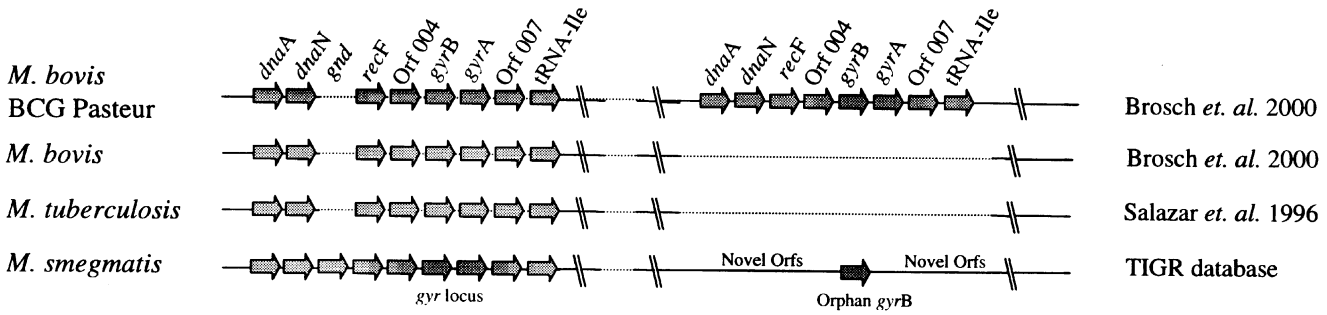

Figure 3. Organization of the *gyr* locus in mycobacteria. Genomic fragment duplication in *M. bovis* (BCG Pasteur) is shown.

GyrB interaction or inactivate GyrB. To overcome this, either production of a resistant GyrB protein or overexpression of the *gyrB* gene is required (as in *E. coli* when treated with cisplatin). One of the *gyrB*s in *M. smegmatis*—either the authentic one, which is part of the *gyrBA* operon, or the orphan *gyrB*—could have been retained as a survival strategy against an antibiotic of unknown nature. Furthermore, the cellular DNA gyrase is a target for proteinaceous poisons encoded by selfish plasmids. For example, CcdB encoded by F plasmid binds to GyrA of *E. coli* and arrests the DNA gyrase reaction at the covalent complex stage (Critchlow *et al.* 1997; Couturier *et al*. 1998). Similarly, microcin B17 encoded by pMccB 17 binds to GyrB and is an effective poison of *E. coli* DNA gyrase (Vizan *et al.* 1991). It is noteworthy that *E. coli* has evolved a counterdefensive strategy of producing GyrI as an antidote to DNA-gyrase-specific proteinaceous poisons (Chatterji and Nagaraja 2002). Although microcin B17 and CcdB do not inhibit *M. smegmatis* DNA gyrase (Chatterji *et al.* 2001), other proteinaceous poisons may be encoded by mycobacterial plasmids. A point to be noted is that *M. smegmatis* is a free-living saprophytic organism with widespread distribution unlike *M. tuberculosis* and hence likely to encounter a variety of plasmids, a large number of *Streptomyces* species, and other bacteria. The additional GyrB could be a mechanism to counter toxins or inhibitors encoded by other genomes.

It is intriguing that different species of mycobacteria have varied GyrB components. What is the physiological basis for this differential distribution? Considering the large difference in the growth rates of *M. smegmatis* and *M. tuberculosis* it is possible that orphan GyrB could be contributing to the higher levels of enzymatic activity required during the log phase of *M. smegmatis* growth in culture. Alternatively, it is expressed differentially under certain conditions as an immediate requirement for cellular function. Another point to be noted is that GyrB is intrinsically less stable than GyrA in *E. coli* (Higgins *et al.* 1978) and also in *M. smegmatis* (unpublished results from our laboratory). The orphan GyrB could be compensating for reduced levels of active GyrB in the cell. Therefore the presence of this additional gene copy in *M. smegmatis* could be a mechanism for regulated synthesis of GyrB under certain conditions. A detailed characterization of the orphan GyrB would be of great importance in revealing its physiological function.

Conclusions

The presence of two genes for GyrB in *M. smegmatis* raises interesting possibilities regarding the intracellular functions of the two proteins. The authentic GyrB is known to be associated with GyrA in a tetrameric holoenzyme that carries out the DNA supercoiling reaction (Manjunatha *et al.* 2002). From our comparative analysis it appears that orphan *gyrB* is a functional allele and hence retained in the genome at a different location. It is possible that mutation in one GyrB may still allow the cell to grow as the other GyrB may provide the required functions. This suggestion is testable by making knockouts of either one of the *gyrB* alleles to evaluate their indispensability.

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