

# Moonlighting function of glutamate racemase from *Mycobacterium tuberculosis*: racemization and DNA gyrase inhibition are two independent activities of the enzyme

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Glutamate racemase (Murl) provides D-glutamate, a key building block in the peptidoglycan of the bacterial cell wall. Besides having a crucial role in cell wall biosynthesis, Murl proteins from some bacteria have been shown to act as an inhibitor of DNA gyrase. *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* Murl exhibit these dual characteristics. Here, we show that the two activities of *M. tuberculosis* Murl are unlinked and independent of each other. The racemization function of Murl is not essential for its gyrase-inhibitory property. Murl–DNA gyrase interaction influences gyrase activity but has no effect on the racemization activity of Murl. Overexpression of Murl *in vivo* provides resistance to the action of ciprofloxacin, suggesting the importance of the interaction in gyrase modulation. We propose that the moonlighting activity of Murl has evolved more recently than its racemase function, to play a transient yet important role in gyrase modulation.

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## INTRODUCTION

The concept of the ‘one gene, one enzyme’ hypothesis, originally proposed by Beadle and Tatum, and later adapted to ‘one gene, one polypeptide’ (Kenez, 1973), has undergone a major modification with the influx of a vast body of information from multiple genomes. It is now apparent that one gene does not necessarily encode only one polypeptide with a single function. Cells employ various strategies to increase protein functionality without the burden of increasing the genome size. One of the means is by evolving ‘moonlighting’ proteins that are able to perform multiple, independent functions (Jeffery, 2003). Moonlighting proteins arise from existing proteins by recruiting existing binding sites for new functions, or by modifying unused regions for a new purpose (Jeffery, 2003). These proteins are usually metabolic enzymes, which in addition to catalysis can perform a second, independent, non-catalytic function. Some can perform both an enzymic and a secondary function simultaneously, while others switch between two functions. However, not all moonlighting proteins are enzymes (Jeffery, 1999).

Moonlighting enzymes can perform their multiple functions by using either the same or distinct binding sites. The

same protein may perform two different functions when located in different parts of a cell. PutA, an enzyme from *Escherichia coli*, acts as a pyrroline-5-carboxylate proline dehydrogenase when associated with the plasma membrane of a cell. However, when it is free in the cytoplasm, it has no enzymic activity, but instead binds DNA and acts as a transcriptional repressor of proline-utilization genes (Lee *et al.*, 2003). Bacterial Cpn10, Cpn60 and Hsp70 are also considered as examples of moonlighting proteins as they have been reported to stimulate or inhibit the proinflammatory actions of myeloid cells and vascular endothelial cells (Henderson, 2005). DegP (HtrA) is a conserved extracytoplasmic *E. coli* heat-shock protein with protease (proteolysis) and chaperone (refolding) activities (Lipinska *et al.*, 1990; Spiess *et al.*, 1999). The protein can monitor the folded state of a substrate protein and then degrade or refold it. It also can switch between chaperone and protease functions in a temperature-dependent fashion; increasing the temperature causes an increase in the protease activity (Lipinska *et al.*, 1990; Spiess *et al.*, 1999). In mammals, phosphoglucose isomerase (PGI), a glycolytic enzyme, is secreted by several cell types and moonlights as neuroleukin (Faik *et al.*, 1988), autocrine motility factor (AMF) (Watanabe *et al.*, 1996) and differentiation and maturation mediator (DMM) (Xu *et al.*, 1996).

Glutamate racemase (MurI) is an essential enzyme involved in the cell wall biosynthesis pathway in bacteria. It catalyses the interconversion of L-glutamate to

**Abbreviations:** AMF, autocrine motility factor; CD, circular dichroism; PGI, phosphoglucose isomerase.

Three supplementary figures are available with the online version of this paper.

D-glutamate, an important building block in peptidoglycan. In the recent past, besides the racemization function, a few glutamate racemases have been shown to moonlight as gyrase inhibitors. *E. coli* MurI was the first one to be shown to inhibit gyrase but only in the presence of UDP-N-acetylmuramyl-L-alanine, a peptidoglycan precursor and an absolute activator for the racemase (Ashiuchi *et al.*, 2002). Subsequently, YrpC, one of the two glutamate racemases from *Bacillus subtilis*, was shown to influence the activity of DNA gyrase in precursor-independent fashion (Ashiuchi *et al.*, 2003). MurI from *Mycobacterium tuberculosis* was shown to inhibit DNA gyrase by preventing DNA-binding activity of the enzyme (Sengupta *et al.*, 2006). Gyrase inhibition thus seems to be an additional property of these enzymes. The observed bifunctionality of glutamate racemases poses several questions. (a) Is the racemization function of MurI necessary for its gyrase inhibition property, i.e. do the two activities mutually influence each other or are they completely independent? (b) Does MurI use the same active site (like PGI described above), i.e. its racemization active site, for gyrase inhibition? (c) What is the effect of the presence of the racemization substrate, L/D-glutamate, on its gyrase-inhibitory function? (d) Why would glutamate racemase, an apparently unlinked enzyme that is involved in cell wall biosynthesis, inhibit DNA gyrase, an essential enzyme involved in the topological transactions of DNA?

In the present study we demonstrate that racemization and gyrase inhibition are two independent activities of *M. tuberculosis* MurI. Using engineered mutants compromised in the racemization function, we show that their gyrase inhibition profiles remain unaffected. Moreover, when expressed *in vivo*, both wild-type and the mutant MurI result in a relaxed genome owing to their gyrase-inhibitory abilities. More importantly, expression of *M. tuberculosis* MurI *in vivo* confers resistance to the action of ciprofloxacin.

## METHODS

**Bacterial strains and plasmids.** *Escherichia coli* strains DH5 $\alpha$  and BL26(DE3) were used for cloning and overexpression, respectively, of *Mycobacterium tuberculosis* MurI mutants. The *murI* gene of *M. tuberculosis* was mobilized in the pJAM2 vector (Triccas *et al.*, 1998) from the pET11d-*murI* construct (Sengupta *et al.*, 2006) to generate pJAM2-*mtmurI*. *Mycobacterium smegmatis* mc<sup>2</sup>155 was used to express MurI in a mycobacterial system. Plasmids pBR322 and pUC18 were used for the biochemical assays.

**Enzyme and substrate preparation.** *E. coli* DNA gyrase subunits GyrA and GyrB were purified as described by Maxwell & Howells (1999). *M. smegmatis* DNA gyrase was purified as described previously (Manjunatha *et al.*, 2002). Supercoiled pUC18 and pBR322 were prepared by standard DNA purification protocols (Sambrook *et al.*, 1989).

**Site-directed mutagenesis and purification of MurI mutants.** Active-site mutants of MurI were generated by site-directed mutagenesis using the megaprimer-inverse PCR method (Kirsch &

Joly, 1998). For generation of the C75S and C185S mutants, a pET20b construct harbouring the wild-type *murI* gene was used as template. The T7 promoter primer was used as forward primer for the C75S mutant and the T7 terminator primer was used as reverse primer for the C185S mutant. Oligonucleotide primers carrying the mutant codon were used as forward primer for the C185S mutant (5'-GCTCTACGCACTATCCACTG-3') and reverse primer for the C75S mutant (5'-GTTGCTAGCGATCACCAACGC-3'). The megaprimers thus generated were used as complementary primers for the second round of PCR amplification. The plasmid harbouring the C75S mutant and C185S primer were used for generating the double mutant. The mutants were confirmed by generation or loss of restriction sites and also by sequencing. The double mutant construct was also mobilized in pJAM2 vector for *in vivo* studies. The MurI mutants were overexpressed from pET20b constructs in *E. coli* BL26(DE3). The proteins were purified as described previously (Sengupta *et al.*, 2006).

**Racemization activity.** The racemization activity of MurI was assessed as described previously (Gallo & Knowles, 1993). MurI samples were incubated in the presence of D-glutamate (Sigma) and then rapidly heated to inactivate the enzyme and assayed for L-glutamate (Sigma) using NAD<sup>+</sup>/L-glutamate dehydrogenase (Sigma) (Sengupta *et al.*, 2006). In another independent assay, varying concentrations of MurI (wild-type, C75S, C185S and C75SC185S double mutant) were incubated in the presence of either D- or L-glutamate substrate (10 mM) in a buffer containing 10 mM Tris/HCl, pH 8.0, and 4 mM  $\beta$ -mercaptoethanol at 30 °C for 30 min. The reactions were stopped by heat inactivation and centrifuged at 13 000 r.p.m. for 10 min to remove any precipitate. The supernatant was then taken and the ellipticity was measured at 204 nm using a CD spectrophotometer. The ellipticity of L/D-glutamate alone was used as a reference and change in the ellipticity was calculated by subtracting the value obtained at the zero time point from the value obtained at the final time point.

**Gyrase assays.** Supercoiling assays for DNA gyrase were carried out at 37 °C with 300 ng relaxed pUC18 and 10 nM DNA gyrase from *M. smegmatis* in supercoiling buffer [35 mM Tris/HCl pH 7.5, 5 mM MgCl<sub>2</sub>, 25 mM potassium glutamate, 2 mM spermidine, 2 mM ATP, 50 mg BSA l<sup>-1</sup> and 90 mg yeast tRNA l<sup>-1</sup> in 5% (v/v) glycerol]. Relaxation assays were carried out with 150 nM *E. coli* gyrase using supercoiled pBR322 as the substrate in the supercoiling buffer devoid of ATP. In order to assess the effect of L/D-glutamate on MurI-mediated inhibition of DNA gyrase, 100 mM of L-glutamate, D-glutamate or potassium chloride was added in the supercoiling assay buffer. The reactions were carried out either in the presence or absence of MurI for 60 min at 37 °C and terminated with 0.6% (w/v) SDS. The assay mixtures were resolved on 1% agarose gel in 40 mM Tris/acetate buffer containing 1 mM EDTA.

**Nucleoid staining.** *M. smegmatis* cells harbouring pJAM2-*murI* constructs (both wild-type and double mutant) were grown in Middlebrook 7H9 medium at 37 °C to mid-exponential phase, then induced with 2% (w/v) acetamide and grown for a further 6 h. In another set, after growth to mid-exponential phase, *M. smegmatis* cells were treated with novobiocin (10  $\mu$ g ml<sup>-1</sup>) for 3 h. Cells were pre-fixed in PBS, 1% (v/v) Triton X-100 and 2% (v/v) toluene solution and incubated overnight at 4 °C. This cell suspension was spotted on a slide and excess solution was removed, followed by the addition of 2 mg lysozyme ml<sup>-1</sup> (freshly prepared) and 10 min incubation. Excess lysozyme was removed by repeated washes with PBS. The slide was dried at room temperature, and then 10  $\mu$ l poly-L-lysine (Sigma) was spread over the sample followed by drying again to fix all cells tightly to it. DAPI solution (4',6-diamidino-2-phenylindole), which binds specifically to DNA, was dropped on the sample and it was covered by a glass coverslip. The nucleoid structure of the

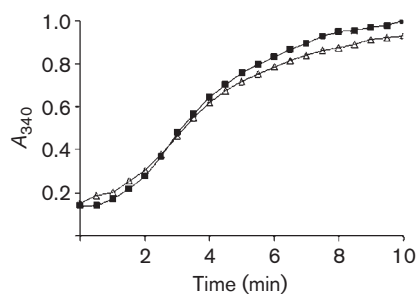
cells was observed through a fluorescence microscope at 60× magnification.

**In vivo cytotoxicity assays.** The effect of MurI on the toxicity caused by ciprofloxacin was tested by spotting various concentrations of the drug on a lawn of *M. smegmatis* mc<sup>2</sup>155 containing either pJAM2 vector or pJAM2-*mtmurI* constructs. *M. smegmatis* transformants were grown in Middlebrook 7H9 medium supplemented with 0.4% (w/v) glucose at 37 °C to mid-exponential phase and then 1% (v/v) inocula were poured onto Middlebrook 7H9 soft agar plates (0.8%, w/v agar) containing appropriate antibiotics. Equal volumes (2 µl) of the drugs from stock solutions of various concentrations were spotted on the lawn of cells and the diameters of the zones of inhibition were measured.

## RESULTS

### DNA gyrase does not influence the racemization function of MurI

In our previous studies we established the mechanism of inhibition of DNA gyrase by MurI (Sengupta *et al.*, 2006). MurI from *M. tuberculosis* interacts with the GyrA subunit of DNA gyrase, which is the primary DNA-binding subunit of the holoenzyme, also responsible for DNA cleavage and religation (Sengupta *et al.*, 2006). The binding of MurI to GyrA thus disturbs the DNA binding and affects catalytic activity of the holoenzyme. We asked whether DNA gyrase has any influence on MurI racemization activity. In order to assess this reciprocal role, MurI was incubated with D-glutamate in either the absence or presence of DNA gyrase, and L-glutamate formed as a result of the racemization reaction was measured by adding NAD<sup>+</sup> and L-glutamate dehydrogenase (GDH). GDH-mediated conversion from L-glutamate to α-ketoglutarate resulted in the reduction of NAD<sup>+</sup> to NADH, which in turn led to an increase in absorbance at 340 nm. As shown in Fig. 1, the racemization activity of MurI remained unaffected upon inclusion of DNA gyrase in the assay mixture. From these results we conclude that the racemization activity of MurI is not influenced by DNA gyrase.



**Fig. 1.** Effect of DNA gyrase on racemization activity of MurI. The racemization assay was carried out as described in Methods, in the absence (■) and presence (△) of DNA gyrase. Reduction of NAD to NADH during the course of the reaction was monitored by measuring absorbance at 340 nm.

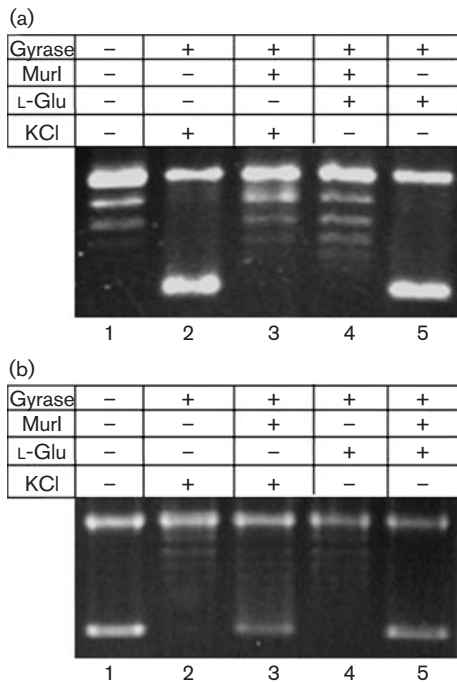
### Racemization substrates have no effect on gyrase inhibition by MurI

Specific binding of MurI to the GyrA subunit prevents the binding of DNA gyrase holoenzyme to DNA (Sengupta *et al.*, 2006). Thus, although protein–protein interaction is necessary for gyrase inhibition, it was not clear whether the racemization activity of MurI has any role in gyrase inhibition. In the case of mammalian PGI, presence of inhibitors or its natural substrates blocked its moonlighting function as an autocrine receptor, thereby suggesting the use of the same active site for both the activities (Watanabe *et al.*, 1996). Further, site-directed mutagenesis of active-site residues needed for PGI activity impaired AMF activity (Suzuki *et al.*, 2002; Tanaka *et al.*, 2002). On the other hand, the moonlighting role of the electron-transport protein cytochrome *c* in apoptosis is not affected by the disruption of the normal function of the protein (Lim *et al.*, 2002). To test the scenario in MurI, we included the racemization substrate (L-glutamate) in the gyrase inhibition assay. Standard gyrase assay buffer contains 25 mM L-glutamate, which was omitted in this case and instead 100 mM of either L-glutamate (potassium salt) or potassium chloride (control) was added. The gyrase-inhibitory profile of MurI remained unaltered irrespective of the presence of the racemization substrate in both the supercoiling (Fig. 2a, compare lanes 3 and 4) and the relaxation assay (Fig. 2b, compare lanes 3 and 5). Based on these results, we infer that MurI probably uses two distinct non-overlapping sites for its two functions; the two activities of MurI seem to be unlinked and independent of each other.

### MurI mutants compromised in the racemization function retain gyrase-inhibition ability

The racemase reaction catalysed by MurI requires an initial deprotonation of the substrate's R-proton, followed by a reprotonation on the opposite face of the resulting planar anionic intermediate (Gallo *et al.*, 1993). In the general mechanism assumed for the reactions catalysed by the pyridoxal 5'-phosphate (PLP)-independent amino acid racemases, two optimally located cysteines are considered to act as catalytic acid/base residues in the two proton transfers required to invert the amino acid's stereochemistry (Fig. 3a) (Glavas & Tanner, 1999, 2001). Primary sequence alignment of *M. tuberculosis* MurI with other glutamate racemases shows two highly conserved signature motifs and cysteine residues at 75 and 185 position emerge as the putative catalytic residues (Supplementary Fig. S1, available with the online version of this paper). These residues were mutated to serine, as it had been shown earlier that such mutations resulted in 1000-fold reduction in the racemization activity of MurI from *Lactobacillus fermenti* (Glavas & Tanner, 1999). In addition to single point mutants, a double mutant was also generated where both cysteines were changed to serines. Mutant MurI proteins (C75S, C185S and C75SC185S double mutant) were purified as described in Methods (Fig. 3b). The





**Fig. 2.** Inhibition of DNA gyrase activities by MurI in the presence of racemization substrate. (a) Effect of presence of L-glutamate on supercoiling activity of DNA gyrase. *M. smegmatis* DNA gyrase at a concentration of 10 nM, with 1  $\mu$ M of either BSA or MurI, was used for the supercoiling reaction. Lanes: 1, relaxed pUC18 alone; 2, gyrase activity in the presence of BSA and 100 mM KCl; 3, gyrase activity in the presence of MurI and 100 mM KCl; 4, gyrase activity in the presence of BSA and 100 mM L-glutamate; 5, gyrase activity in the presence of MurI and 100 mM L-glutamate. (b) Relaxation activity of DNA gyrase. *E. coli* enzyme at a concentration of 150 nM was used. Lanes: 1, supercoiled pUC18 alone; 2, DNA gyrase activity in the presence of BSA and 100 mM KCl; 3, gyrase activity in the presence of MurI and 100 mM KCl; 4, gyrase activity in the presence of BSA and 100 mM L-glutamate; 5, gyrase activity in the presence of MurI and 100 mM L-glutamate. All the assays were repeated at least three times. Representative results are presented.

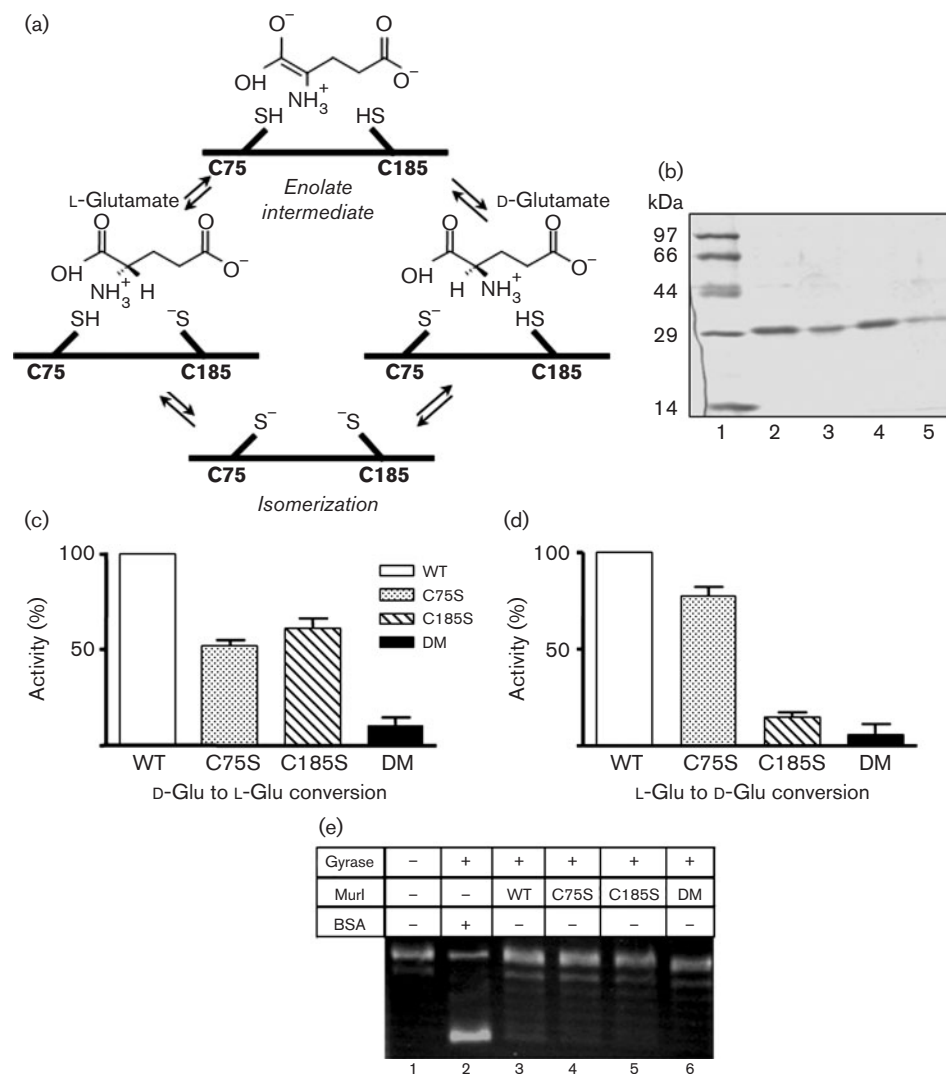
racemization activities of these mutants were monitored by CD spectroscopy. The mutants were defective in racemization function (both L to D as well as D to L conversion) to a varying degree and the double mutant (C75SC185S) retained very low (less than 10%) racemization activity (Fig. 3c, d). Next, the mutants were assessed for their ability to inhibit gyrase. All the mutants exhibited similar gyrase-inhibitory profiles, comparable to that of the wild-type (Fig. 3e). Even the double mutant, which was highly compromised in its racemization function (Fig. 3c, d), retained its gyrase inhibition ability, comparable to the wild-type enzyme (Fig. 3e, lane 6). From these results, we conclude that the two activities of this bifunctional MurI are unlinked and independent of each other.

### Alterations in the nucleoid structures upon overexpression of MurI

DNA gyrase is involved in the maintenance of the negative supercoiling status of the genome. Since MurI inhibits DNA gyrase activity, it might play a role in modulation of the topological status of the genome. In order to assess this property, we monitored the nucleoid status in *M. smegmatis* cells after overexpression of MurI. Both the wild-type and the double mutant forms of MurI were overexpressed from pJAM2 constructs in *M. smegmatis* (Supplementary Fig. S2). In a parallel experiment, *M. smegmatis* cells were treated with another well-known gyrase inhibitor, novobiocin, at a concentration which has been shown to result in genomic relaxation (Unniraman & Nagaraja, 1999). As shown in Fig. 4 and also Supplementary Fig. S3, the nucleoids in bacteria overexpressing MurI (wild-type as well as mutant) appeared to be diffused and dispersed throughout the entire length of the cell, resembling the nucleoid status observed in novobiocin-treated cells. In contrast, the cells harbouring the vector alone showed a rather compact nucleoid structure at the mid-cell site. These results indicate that both the wild-type and the mutant MurI were able to inhibit the supercoiling activity of DNA gyrase *in vivo*. These studies further corroborate the *in vitro* data showing that the double mutant with a compromised racemization activity still retained its gyrase inhibition ability *in vivo*, resulting in the relaxed genomic status.

### MurI protects the bacteria from the action of ciprofloxacin

The above results establish that moonlighting function of MurI in gyrase modulation has originated independent of its racemization property. What would be the physiological basis for evolution of such an unlinked property in a dedicated cell wall precursor synthesizing enzyme? One possibility is that interaction of MurI with gyrase protects it from the action of other inhibitors. In order to address this, we spotted different concentrations of ciprofloxacin on a lawn of *M. smegmatis* cells overexpressing the wild-type or double mutant form of MurI. The formation of a zone of inhibition and its size is indicative of the sensitivity of the cells to ciprofloxacin. Diameters of the zones of inhibition were measured (Table 1). The reduced diameters of the zones of inhibition in the case of MurI-overexpressing cells indicate that MurI expression leads to increased resistance to ciprofloxacin. MurI reduced the double strand breaks induced by ciprofloxacin *in vitro* (Fig. 5). MurI did not provide protection against mitomycin C, a general DNA-damaging agent, thereby ruling out the possibility that the ciprofloxacin resistance might be due to altered cell wall permeability (not shown). MurI-mediated protection was thus specific to DNA damage caused by trapped gyrase-DNA covalent complexes. The double mutant compromised in its racemization function protected against the action of ciprofloxacin in a similar manner to that of wild-type enzyme.

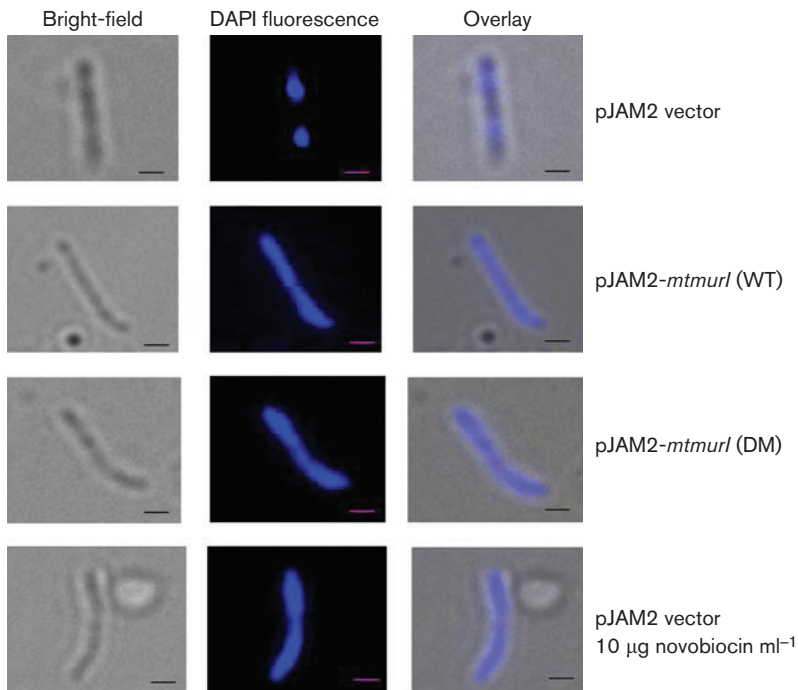


**Fig. 3.** Analysis with site-directed mutants of *M. tuberculosis* MurI. (a) Proposed catalytic mechanism of glutamate racemase. Conserved putative active-site cysteines (*M. tuberculosis*) are shown schematically. Isomerization of the enzyme is represented proceeding through the fully deprotonated state. (b) SDS-PAGE profile of purified mutants of *M. tuberculosis* MurI. Lanes: 1, size markers; 2, wild-type MurI; 3, C75S MurI; 4, C185S MurI; 5, C75SC185S MurI. (c, d) CD spectrometry showing the comparative racemization activity of these mutants, i.e. L-glutamate to D-glutamate conversion and vice versa. MurI samples (1  $\mu$ M) were incubated in the presence of either D- or L-glutamate substrate (10 mM) in buffer as described in Methods at 30 °C for 30 min. The reactions were stopped and the change in ellipticity was measured at 204 nm using a CD spectrophotometer. Activity obtained with the wild-type enzyme was taken as 100% and the relative percentage activities of the mutants were then plotted. (e) DNA gyrase supercoiling assay with these mutants. DNA gyrase at a concentration of 10 nM, with 1  $\mu$ M of BSA or MurI, was used for the assay. Lanes: 1, relaxed pUC18; 2, gyrase with BSA; 3, gyrase with wild-type MurI; 4, gyrase with C75S MurI; 5, gyrase with C185S MurI; 6, gyrase with C75SC185S double mutant MurI.

## DISCUSSION

In the present study, we have shown that the two activities of MurI from *M. tuberculosis*, viz. racemization and gyrase inhibition, are unlinked and independent of each other. Using site-directed mutants compromised in the racemization function, we have demonstrated that the racemization activity is not required for the gyrase-modulatory role of

MurI. This also suggests that probably a different surface of MurI, away from its active site, is involved in interacting with gyrase. The existence of a glutamate racemase that does not inhibit gyrase, i.e. Glr from *B. subtilis* (Ashiuchi *et al.*, 2003), provides further support in this regard, since in Glr, the racemization domains are highly conserved and similar to those found in glutamate racemases having gyrase-inhibitory properties. *M. tuberculosis* MurI interacts



**Fig. 4.** Overexpression of MurI results in diffused and dispersed nucleoid structures. Comparison of nucleoid structures in *M. smegmatis* mc<sup>2</sup>155 cells harbouring either the pJAM2 vector or pJAM2-*mtmurI* (wild-type, WT) or pJAM2-*mtmurI* (double mutant, DM) constructs induced with 2% acetamide. Single bacterial cells have been enlarged from a group of cells of similar phenotype. Novobiocin-treated cells were used as positive control, showing the relaxed diffused nucleoids of cells treated with the coumarin class of gyrase inhibitor. Left panels, bright-field images; middle panels, fluorescent images showing the DAPI-stained nucleoid; right panels, overlay of the two images. Scale bars, 1  $\mu\text{m}$ .

with the DNA-binding subunit of DNA gyrase, GyrA (Sengupta *et al.*, 2006). Its interaction with GyrA thus perturbs the normal catalytic activity of the gyrase, which involves DNA binding followed by cleavage and religation. In contrast, the gyrase interaction surface on MurI does not seem to overlap with the catalytic site of the latter.

The physiological significance of MurI-mediated gyrase modulation is yet to be understood. By preventing the DNA-binding activity of gyrase, it protects the enzyme against the cytotoxic action of gyrase poisons such as ciprofloxacin. These observations could indicate that MurI may function to safeguard the essential housekeeping enzyme, DNA gyrase, from gyrase-targeting poisons. Unlike in *E. coli*, addiction

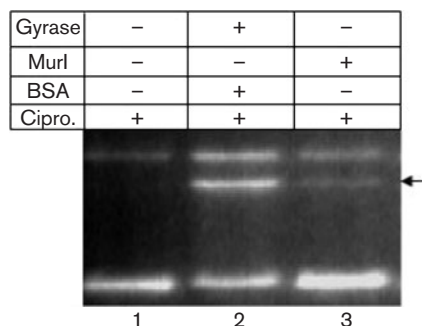
modules which target DNA gyrase in mycobacteria have not been characterized so far. Alternatively, under conditions when gyrase activity needs to be kept under control, MurI could be involved in sequestration of DNA gyrase away from its site of action, thus serving as a 'checkpoint' coordinating the cell division and DNA replication.

An alarming increase in the emergence of multidrug-resistant strains of *M. tuberculosis* has led to an active search for novel drug targets. MurI is an attractive target for designing new antibacterial drugs as it provides D-glutamate, an important component for peptidoglycan biosynthesis (Lundqvist *et al.*, 2007). Overexpression of *M. tuberculosis* MurI in soluble form and also in large quantities described here

**Table 1.** MurI expression increases resistance to ciprofloxacin

Cultures of *M. smegmatis* harbouring either the vector pJAM2 or pJAM2-*mtmurI* constructs (wild-type or double mutant) were grown in Middlebrook 7H9 broth to mid-exponential phase at 37 °C and then poured onto soft agar plates to form a lawn. Different dilutions of ciprofloxacin were spotted and then the plates were incubated for 3–4 days to get defined zones of inhibition.

Ciprofloxacin ( $\mu\text{g}$ )	Zone of inhibition (mm)		
	pJAM2 vector	pJAM2- <i>mtmurI</i> (wild-type)	pJAM2- <i>mtmurI</i> (double mutant)
0.5	10 $\pm$ 0.4	6.5 $\pm$ 0.2	6.2 $\pm$ 0.3
0.45	7.5 $\pm$ 0.3	5.5 $\pm$ 0.6	5.7 $\pm$ 0.2
0.4	5.5 $\pm$ 0.5	3.5 $\pm$ 0.7	4.1 $\pm$ 0.4
0.35	3.5 $\pm$ 0.4	1.5 $\pm$ 0.3	1.7 $\pm$ 0.6
0.3	2 $\pm$ 0.2	–	–
0.25	1 $\pm$ 0.4	–	–



**Fig. 5.** Effect of MurI on the ciprofloxacin-induced cleavage reaction. DNA gyrase at a concentration of 100 nM was used for the assays. Lanes: 1, supercoiled pUC18; 2, gyrase with 2  $\mu$ M BSA; 3, gyrase with 2  $\mu$ M MurI. A ciprofloxacin concentration of 30  $\mu$ g ml<sup>-1</sup> was used in the assays.

(Supplementary Fig. S2) would facilitate high-throughput screening of antimycobacterial compounds targeting MurI.

Finally, why have enzymes like MurI evolved such a distinct moonlighting activity? Multifunctional proteins, which are mostly ubiquitous enzymes, have acquired some additional functions over the long period of their existence. Several enzymes appear to be much larger than what seems to be necessary for performing a single function. They often have large unused solvent-exposed areas and pockets on the protein surface that could be modified to make additional binding sites. As long as these additional functions do not interfere with the original function of the protein, they might benefit the cell by providing a competitive advantage during evolution. By having multifunctional proteins, a cell has fewer proteins to synthesize and, consequently, less DNA to replicate, thereby saving a great deal of energy in growth and reproduction (Jeffery, 1999, 2003). Moonlighting proteins can also provide a means of coordinating or modulating cellular activities as in the case of MurI. The increasing number of proteins that are being found to moonlight adds another level to cellular complexity and regulation.

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