RESEARCH LETTER

Inhibition of DNA gyrase activity by *Mycobacterium smegmatis* MurI

Sugopa Sengupta[1] & Valakunja Nagaraja[1,2]

[1]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India; and [2]Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India

Correspondence: Valakunja Nagaraja, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India. Tel.: +91 80 2360 0668; fax: +91 80 2360 2697; e-mail: vraj@mcbl.iisc.ernet.in

Received 10 September 2007; accepted 29 October 2007.
First published online December 2007.

DOI:10.1111/j.1574-6968.2007.01005.x

Editor: Roger Buxton

Keywords
Mycobacterium smegmatis; glutamate racemase; DNA gyrase; gyrase inhibitor; protein–protein interaction.

Abstract

Glutamate racemase (MurI) catalyzes the interconversion of L-glutamate to D-glutamate, one of the essential amino acids present in the peptidoglycan. In addition to this essential enzymatic function, MurI from *Escherichia coli, Bacillus subtilis* and *Mycobacterium tuberculosis* inhibit DNA gyrase activity. A single gene for *murI* found in the *Mycobacterium smegmatis* genome was cloned and overexpressed in a homologous expression system to obtain a highly soluble enzyme. In addition to the racemization activity, *M. smegmatis* MurI inhibits DNA gyrase activity by preventing DNA binding of gyrase. The sequestration of the gyrase by MurI results in inhibition of all reactions catalyzed by DNA gyrase. More importantly, MurI overexpression *in vivo* in mycobacterial cells provides protection against the action of ciprofloxacin. The DNA gyrase-inhibitory property thus appears to be a typical characteristic of MurI and would have probably evolved to either modulate the function of the essential housekeeping enzyme or to provide protection to gyrase against gyrase inhibitors, which cause double-strand breaks in the genome.

Introduction

All bacteria obtain their structural integrity from the peptidoglycan layer, a large polymeric structure that is located between the cytoplasmic and the outer membranes of the cell wall. Peptidoglycan is formed from linear chains of two alternating amino sugars, namely *N*-acetylglucosamine and *N*-acetylmuramic acid, to which a small group of amino acids (stem peptide) consisting of L-alanine, D-alanine, D-glutamate and either lysine or diaminopimelic acid are covalently attached (Meroueh *et al*., 2006). Glutamate racemase (MurI) catalyzes the interconversion of L-glutamate to D-glutamate, one of the integral components present in peptidoglycan. It is ubiquitously inherited in bacteria although with undetectable activity, except in some lactobacilli (Gallo & Knowles, 1993).

By virtue of being unique to bacteria, peptidoglycan and the enzymes involved in its biosynthesis have been considered to be attractive targets for the design and development of new antibacterial agents. Approaches in this direction include rational, structure-based drug design to inhibit the synthesis of the stem peptide portion of the peptidoglycan precursor (Tanner *et al*., 1996; Zeng *et al*., 1998; Reck *et al*., 2001). MurI, a provider for D-glutamate, has been shown to be important in bacteria including pathogenic species (Hoskins *et al*., 1999; Glavas & Tanner, 2001). Being an essential enzyme, it is already used as a target for antibacterial drug discovery. The first discovered, moderately potent MurI inhibitors competitively interfered with the L/D transformation (Tanner & Miao, 1994; Glavas & Tanner, 1997; de Dios *et al*., 2002). The design of D-glutamate-based analogues and their structure activity relationship studies led to the discovery of several potential MurI inhibitors (Glavas & Tanner, 1997; de Dios *et al*., 2002).

Besides the racemization function, few glutamate racemases have been shown to possess an additional property of gyrase inhibition. *Escherichia coli* MurI was the first one to be shown to inhibit *E. coli* gyrase, but only in the presence of the peptidoglycan precursor (Ashiuchi *et al*., 2002). Subsequently, one of the two glutamate racemases from *Bacillus subtilis*, the YrpC isozyme, was shown to influence the activity of DNA gyrase in a precursor independent fashion (Ashiuchi *et al*., 2003). MurI from *Mycobacterium tuberculosis* inhibits DNA gyrase by preventing the DNA-binding

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

activity of the enzyme (Sengupta *et al*., 2006). Gyrase inhibition seems to be an additional attribute of some of the glutamate racemases, but not all, as Glr isozyme from *B. subtilis* has no effect on gyrase activity (Ashiuchi *et al*., 2003).

MurI from *M. tuberculosis* cloned in *E. coli* was found to be in the insoluble inclusion bodies (Sengupta *et al*., 2006). Attempts to express the protein in soluble form in *E. coli* were unsuccessful. The active enzyme was recovered after urea refolding and was then used for biochemical characterization. It is important to obtain the protein in soluble form and in large quantities to carry out structural studies and detailed characterization.

Like *M. tuberculosis,* the *Mycobacterium smegmatis* genome contains a single gene for glutamate racemase although the genome of the latter is larger. Understanding the characteristics of *M. smegmatis* MurI is important because *M. smegmatis* is often used as a surrogate for *M. tuberculosis* for a variety of studies. Comparison of the properties of MurI from both the species would form the basis for its use for *in vivo* screening of antimycobacterial compounds.

In the present study, *M. smegmatis* MurI has been expressed and purified in soluble form with high specific activity. The studies reveal that MurI from *M. smegmatis* is also a bifunctional enzyme. It inhibits DNA gyrase in addition to its racemization activity. Further, overexpression of the enzyme in *M. smegmatis* provides protection to the organism against fluoroquinolones.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strains DH5α and *M. smegmatis* mc^2155 strains were used for cloning and overexpression of *M. smegmatis* MurI, respectively. Genomic DNA from *M. smegmatis* mc^2155 was isolated as described (Madhusudan *et al*., 1994). The *murI* gene was cloned in the pJAM2 vector (Triccas *et al*., 1998). pBR322 and pUC18 plasmids were used for the biochemical assays.

Enzyme and substrate preparation

Escherichia coli DNA gyrase subunits, GyrA and GyrB, were purified as described (Maxwell & Howells, 1999). *Mycobacterium smegmatis* DNA gyrase was purified by the method standardized before (Manjunatha *et al*., 2002). Supercoiled pUC18 and pBR322 were prepared by standard protocols (Sambrook *et al*., 1989).

Cloning of *murI*

The *murI* gene was PCR amplified using *M. smegmatis* mc^2 genomic DNA as a template and primers Pf (5′-TCTGAC CATATGAGCGATCGACT-3′) and Pr (5′-GGTTTCGCGA CACAGATCATCTTC-3′) containing NdeI and NruI sites, respectively. The gene was amplified with Pfu DNA polymerase. In brief, 50 ng of genomic DNA was processed in a 50 μL reaction volume containing PCR buffer [20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg mL^{-1} bovine serum albumin (BSA)], 250 μM deoxynucleoside triphosphates, 20 pmol of each primer and 1 U of *Pfu* polymerase (Promega). Amplifications were carried out in a GeneAmp PCR system 9700 of Applied Biosystems. The first cycle, consisting of a 5-min denaturation at 94 °C, was followed by 30 cycles each of 1 min at 94 °C, 45 s at 56 °C, and 2 min, 30 s, at 68 °C, with a final extension for 5 min at 68 °C.

The amplicon was subjected to NdeI digestion, followed by end filling with Klenow polymerase, and ligated to an ScaI digested pJAM2 vector to generate a pJAM2-*msmurI* construct.

Expression and purification of MurI

The *M. smegmatis* mc^2155 cells harboring the pJAM2-*msmurI* construct were grown in Middle brook 7H9 media. The cells were harvested, resuspended in sonication buffer containing 50 mM Tris-HCl pH-8.0, 1 mM potassium glutamate, 0.1 mM phenyl methane sulfonyl fluoride, 0.5 mM $MgCl_2$, and sonicated. Lysis was followed by ultracentrifugation at 100 000 ***g*** at 4 °C for 3 h. The supernatant was subjected to 60% ammonium sulfate fractionation. The pellet containing MurI was dialyzed against buffer A (50 mM Tris-HCl pH-8.0, 0.2% 2-mercaptoethanol, 0.1 mM phenyl methane sulfonyl fluoride, 10% glycerol) and loaded onto a MonoQ column, washed with buffer A containing 200 mM NaCl and the enzyme was eluted with a gradient of 200–500 mM NaCl. The peak fractions containing MurI were pooled and dialyzed against the same buffer, reloaded onto MonoQ column and eluted with a shallow gradient of 250–350 mM NaCl. The fractions containing MurI were pooled and subjected to 0–60% ammonium sulfate fractionation. The pellet was dissolved in 2 mL of the buffer A, dialyzed and loaded onto a Sephacryl S100 gel filtration column. The eluted fractions containing MurI were pooled, dialyzed, concentrated and stored at − 70 °C.

Racemization activity

The racemization activity was assessed by a coupled assay as described previously (Sengupta *et al*., 2006). MurI was incubated in the presence of D-glutamate for 30 min at 37 °C and then rapidly heated to inactivate the enzyme and further assayed for L-glutamate using NAD^+/L-glutamate dehydrogenase. The increase in $A_{340\,nm}$ was monitored for 10 min at 25 °C using a Beckman DU640 UV/vis spectrophotometer.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

DNA supercoiling and relaxation reactions

Supercoiling assays were carried out at 37 °C with 300 ng of relaxed pUC18 and 10 nM DNA gyrase from either *M. smegmatis* in supercoiling buffer [Buffer S: 35 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 25 mM potassium glutamate, 2 mM spermidine, 2 mM ATP, 50 mg L^{-1} BSA and 90 mg L^{-1} yeast tRNA in 5% (v/v) glycerol]. Relaxation assays were carried out with 150 nM of gyrase using supercoiled pBR322 in buffer S devoid of ATP, either in the presence or in the absence of MurI for 60 min at 37 °C and terminated with 0.6% sodium dodecylsulfate (SDS). The assay mixtures were resolved on 1% agarose gel in 40 mM Tris-actetate buffer containing 1 mM EDTA.

Electrophoretic mobility shift assay (EMSA)

Assays were carried out using an end-labeled 240-bp DNA fragment encompassing the strong gyrase site (SGS) from pBR322. One nanomolar of labeled DNA was incubated with 100 nM of DNA gyrase either in the absence or in the presence of different concentrations of MurI in buffer S for 30 min at 4 °C, followed by electrophoresis on 4% native polyacrylamide gel as described (Sengupta *et al.*, 2006). The free DNA and bound complexes were quantitated using a phosphorimager.

Cleavage reactions

DNA cleavage assays were carried out in buffer S with a supercoiled pBR322 substrate or radiolabeled 240-bp SGS for 30 min at 30 °C, and the gyrase–DNA complex was trapped by adding 0.2% SDS, followed by proteinase K (90 μg mL^{-1}) digestion for 30 min. In case of drug-induced cleavage reactions, ciprofloxacin (30 μg mL^{-1}) was included in the assay. The reaction mixtures were resolved on 1% agarose gel or 8% urea–polyacrylamide gel electrophoresis (PAGE). The substrate and cleaved DNA products were quantitated using a phosphorimager.

ATPase assay

The DNA-stimulated ATPase activity of DNA gyrase was monitored in buffer S containing 2 mM ATP, 10 μg mL^{-1} DNA (240 bp from pBR322) and 0.02 μCi of [γ-^{32}P] ATP (3000 Ci $mmol^{-1}$). In order to assess the intrinsic ATPase activity, 1 μM GyrB was used while DNA and GyrA were omitted. The assays were carried out as described earlier (Sengupta *et al.*, 2006). The aqueous layer (1.0 μL) was resolved on polyethyleneimine-cellulose by thin-layer chromatography using 1.2 M LiCl, and 0.1 mM EDTA as the chase buffer. The spots corresponding to ATP and P_i were quantitated using a phosphorimager.

Growth profiles and cytotoxicity assays

Mycobacterium smegmatis mc^{2}155 cells containing either the pJAM2 vector or the pJAM2-*msmurI* construct were grown in Middlebrook 7H9 media supplemented with 0.4% glucose at 37 °C, and growth was monitored by measuring $A_{600\,nm}$ after every 3 h. The effect of MurI on the toxicity caused by drugs (ciprofloxacin and mitomycin C) was tested by spotting various concentrations of the drug on a lawn of *M. smegmatis* mc^{2}155 containing either the pJAM2 vector or pJAM2-*msmurI* constructs. The cultures were grown to the mid-logarithmic phase and then 1% inoculums were poured onto Middlebrook 7H9-soft agar plates (0.8% agar) containing appropriate antibiotics. The plates were preincubated for drying before the spotting. Equal volumes (2 μL) of drugs from the stock solutions of varying concentrations were spotted on the lawn of cells, and the diameters of the zones of inhibition were measured and tabulated to compare the potency of the drugs for the *M. smegmatis* transformants.

Results

Purification of *M. smegmatis* MurI

The recombinant glutamate racemase was overexpressed from the pJAM2-*msmurI* construct in the *M. smegmatis* mc^{2}155 strain. Constitutive high-level expression was obtained without requiring acetamide-mediated induction (Fig. 1). The identity of the protein was confirmed by the standard tryptic mass fingerprinting technique (not shown). The recombinant protein was found totally in the soluble fraction in contrast to the *M. tuberculosis* enzyme expressed

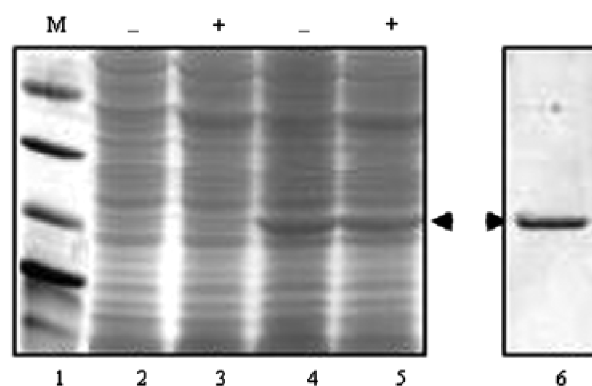

Fig. 1. Characterization of *Mycobacterium smegmatis* MurI. Expression profile of *M. smegmatis* MurI, lane 1 and lane 2: *M. smegmatis* mc^{2}155 cell extract harboring vector pJAM2 vector uninduced and induced with 2% acetamide, respectively, lane 3 and lane 4: *M. smegmatis* mc^{2}155 cell extract harboring pJAM2-*msmurI* construct uninduced and induced with 2% acetamide, respectively, lane 5: purified MurI. –, uninduced; +, induced with acetamide

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

in *E. coli*. It was purified to apparent homogeneity and racemase activity was assayed by the glutamate dehydrogenase coupled assay. The specific activity was determined to be 3 U mg^{-1} of the enzyme, 1 U being defined as the amount of enzyme required to convert 1 μmol of substrate into product min^{-1}. The soluble protein was found to be 10-fold more active when compared with urea-refolded *M. tuberculosis* MurI.

Mycobacterium smegmatis MurI inhibits DNA gyrase activity

Alignment of the *M. smegmatis* MurI sequence with that of the *M. tuberculosis* enzyme revealed that they share 82% identity at the amino acids level. To test the effect of *M. smegmatis* MurI on supercoiling activity, mycobacterial DNA gyrase was preincubated with MurI before the addition of relaxed pUC18. MurI inhibited supercoiling activity of DNA gyrase in a dose-dependent fashion (Fig. 2a). To test the effect of MurI on DNA gyrase-mediated relaxation, DNA gyrase was preincubated with MurI prior to the addition of a supercoiled pBR322 DNA substrate. MurI was found to inhibit the DNA relaxation activity of gyrase as well (Fig. 2b). MurI inhibited the decatenation activity and also the activity of gyrase from a heterologous source i.e. from *E. coli* at comparable levels (not shown). Hence, MurI inhibits DNA gyrase across the species barrier unlike the toxins CcdB and microcin B17 (Chatterji *et al.*, 2001), which are specific inhibitors of *E. coli* DNA gyrase.

MurI inhibits at the step of DNA binding

To assess the effect of MurI on gyrase–DNA complex formation, EMSAs were performed by preincubating DNA gyrase with different concentrations of MurI, prior to the addition of DNA. MurI inhibited the formation of the gyrase–DNA noncovalent complex in a dose-dependent manner (Fig. 3a). If DNA binding by gyrase is the target of MurI action, the DNA-stimulated ATPase activity of the enzyme should be affected while its intrinsic activity should be unaltered. Reactions were carried out in the presence of a linear DNA substrate as described in 'Materials and methods'. MurI inhibited the DNA-stimulated ATPase activity of DNA gyrase (Fig. 3b). However, as expected, the intrinsic ATPase activity of DNA gyrase was not altered in the presence of MurI (Fig. 3c).

To test the effect of MurI at the cleavage step, DNA gyrase was preincubated with MurI and the cleavage assays were performed both with the supercoiled plasmid DNA and the radiolabeled linear DNA substrates. Ciprofloxacin is known to arrest DNA gyrase at the cleavage step and calcium (Ca^{2+}) ions inhibit the religation and stimulate the DNA cleavage activity of the enzyme (Gmunder *et al.*, 1995; Lewis *et al.*, 1996; Maxwell, 1999). SDS and proteinase K treatment removes the covalently attached protein and a cleaved DNA

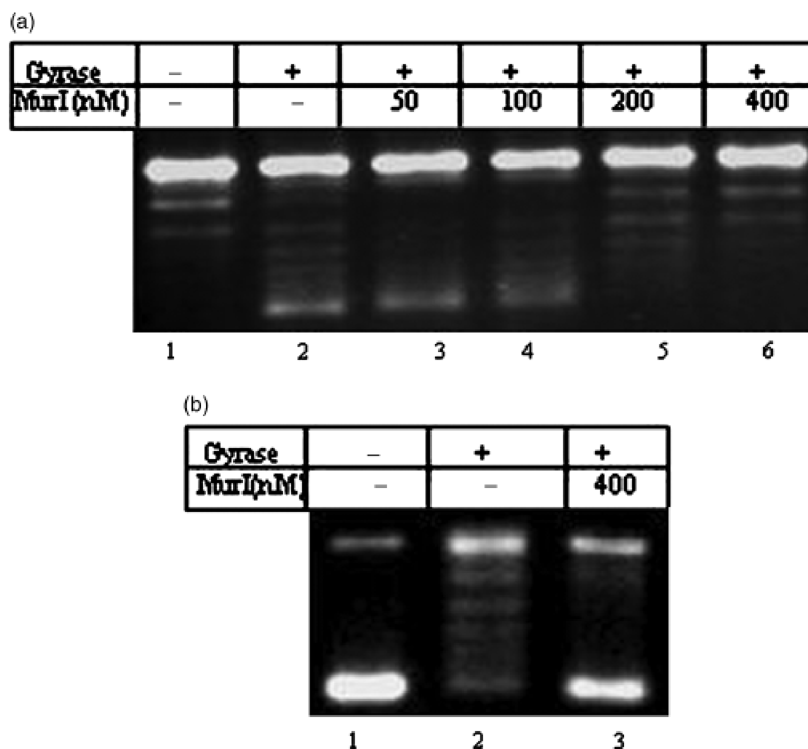

Fig. 2. Inhibition of DNA gyrase activities by *Mycobacterium smegmatis* MurI. (a) Effect on supercoiling activity of *M. smegmatis* DNA gyrase. Ten nanomolars of DNA gyrase was used for the supercoiling reaction; lane 1: relaxed pUC18 alone, lane 2: gyrase activity in the presence of 400 nM BSA, lanes 3–5: gyrase reaction in the presence of increasing concentrations of MurI (50, 100, 200, 400 nM); (b) relaxation activity of DNA gyrase. One hundred and fifty nanomolars of enzyme was used. Lane 1: supercoiled pUC18 alone, lane 2: DNA gyrase activity in the presence of BSA; lane 3: gyrase reaction in the presence of 400 nM MurI. All the assays were repeated at least three times. The representative figures have been presented.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

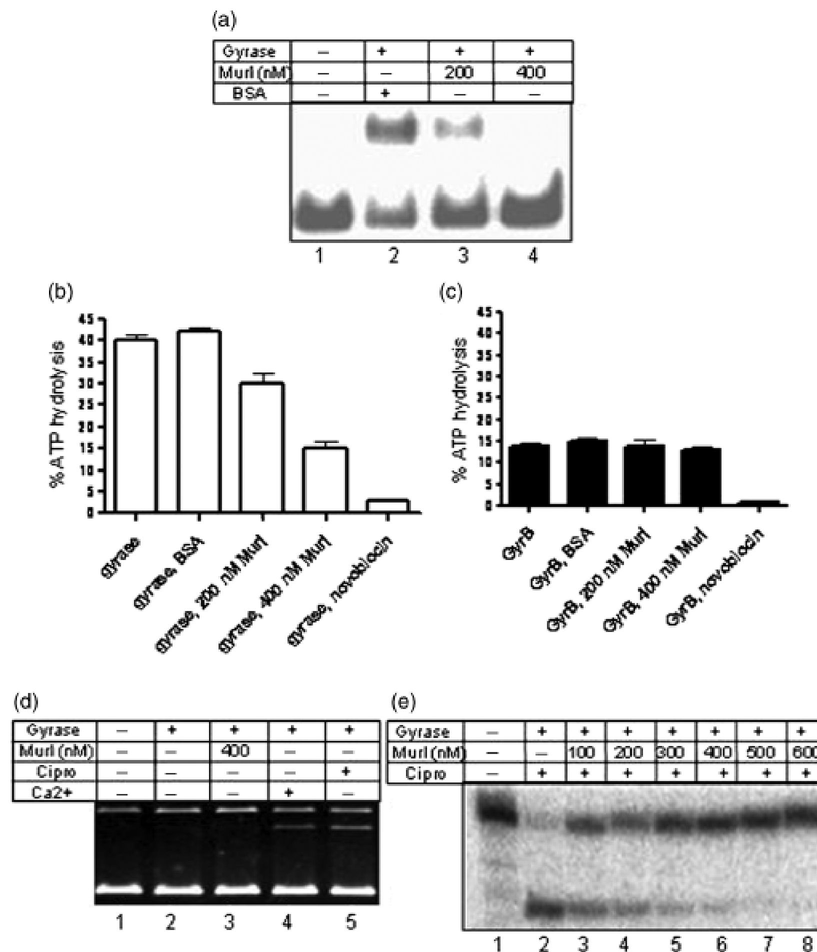

Fig. 3. Effect of MurI on different steps of the gyrase reaction cycle. (a) Effect of MurI on noncovalent complex formation; EMSAs were carried out with 100 nM DNA gyrase and 1 nM radiolabeled 240-bp SGS at 4 °C; lane1: free SGS, lane 2: DNA gyrase in the presence of 400 nM BSA, lanes 3 and 4: gyrase in the presence of 200 and 400 nM of MurI, respectively. (b) DNA stimulated ATPase activity. Reactions were performed with 40 nM *Mycobacterium smegmatis* DNA gyrase and 10 μg mL^{-1} DNA (240-bp SGS from pBR322), lane 1: gyrase alone, lane 2: gyrase in the presence of 400 nM BSA, lanes 3 and 4: gyrase in the presence of 200 and 400 nM of MurI, respectively, lane 5: gyrase in the presence of 1 μg mL^{-1} novobiocin, (c) intrinsic ATPase activity. Reactions were performed with 1 μM *Escherichia coli* GyrB, lane 1: GyrB alone, lane 2: GyrB in the presence of 400 nM BSA, lanes 3 and 4: GyrB in the presence of 200 and 400 nM of MurI respectively, lane 5: GyrB in the presence of 1 μg mL^{-1} novobiocin, (d) cleavage reactions with supercoiled pBR322 substrate, 50 nM DNA gyrase used; lane 1: supercoiled substrate alone, lane 2: DNA gyrase alone, lane 3: gyrase reaction in the presence of 0.4 μM MurI, lane 4: gyrase reaction in the presence of calcium ions, lane 5: gyrase reaction in the presence of ciprofloxacin (30 μg mL^{-1}); Mg^{2+} ions omitted and 5 mM Ca^{2+} ions added in lane 4 (e) cleavage with linear radiolabeled DNA (SGS). 100 nM DNA gyrase used; lane 1: 240-bp SGS alone, lane 2: cleavage by DNA gyrase, lanes 3–8: gyrase mediated cleavage in the presence of increasing concentrations of MurI (100, 200, 300, 400, 500, 600 nM). Ciprofloxacin (30 μg mL^{-1}) added in all the lanes. A representative set of data is presented based on several sets of experiments.

fragment can be visualized on the gel. Unlike ciprofloxacin and calcium ions, MurI did not stimulate DNA gyrase-mediated cleavage (Fig. 3d, compare lanes 3 with lanes 4 and 5). MurI also abrogated ciprofloxacin-induced cleavage on a linear DNA substrate (Fig. 3e). From all these observations, it is concluded that *M. smegmatis* MurI also inhibits the DNA-binding activity of DNA gyrase in a manner similar to that of *M. tuberculosis* MurI. Because, DNA binding is the first step in the catalytic cycle of gyrase, all the subsequent steps are affected in the presence of MurI. Moreover, ciprofloxacin targets the gyrase–DNA covalent complex for stabilization, and MurI interaction with DNA gyrase prevents ciprofloxacin action *in vitro*.

MurI protects the bacteria from the action of ciprofloxacin

Because MurI appears to protect against ciprofloxacin action *in vitro* (Fig. 3d and e), to evaluate the scenario *in vivo*, the cytotoxic action of quinolones was tested by

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

spotting different concentrations of ciprofloxacin on a lawn of *M. smegmatis* cells overexpressing MurI. The formation of a zone of inhibition and its size is indicative of the sensitivity of the cells to ciprofloxacin. The diameters of the zone of inhibition were measured (Table 1). The reduced diameters of zone of inhibition with ciprofloxacin in case of MurI overexpressing cells indicate that MurI overexpression leads to increased resistance against ciprofloxacin. MurI-mediated protection was specific towards DNA damage caused by trapped gyrase–DNA covalent complexes as MurI did not provide protection against mitomycin C, a general DNA-damaging agent (Table 1). These *in vivo* experiments support the *in vitro* data that MurI prevents the DNA-binding activity of gyrase and by doing so, protects gyrase against the cytotoxic action of gyrase poison, ciprofloxacin. The growth profile of *M. smegmatis* overexpressing MurI was compared with the cells harboring only the pJAM2 vector. There were no significant differences in growth upon MurI expression and plasmid topology remained unaltered (not shown). From these results, it appears that MurI-mediated inhibition of DNA gyrase could be transient and gyrase is able to overcome it probably by means of relaxation-stimulated transcription (RST) (Unniraman & Nagaraja, 1999).

Discussion

Mycobacterium smegmatis glutamate racemase exhibits a dual role by possessing racemase activity and DNA gyrase inhibition. Thus, in this respect it is similar to the glutamate racemases from *E. coli*, *M. tuberculosis* and YrpC from *B. subtilis* (Ashiuchi *et al*., 2002, 2003; Sengupta *et al*., 2006). However, unlike the *E. coli* enzyme, the *M. smegmatis* MurI enzyme appears to be active independent of any peptidoglycan precursor as far as racemization as well as gyrase inhibitory properties are concerned. The first report on inhibition of DNA gyrase by glutamate racemase (Ashiuchi *et al*., 2002) was rather surprising as the enzyme appeared to have no role in DNA transaction processes and seemed to be dedicated only to cell wall component biosynthesis. Subsequent studies from the same group with the enzyme from *B. subtilis* (Ashiuchi *et al*., 2003), the authors' studies with *M. tuberculosis* MurI (Sengupta *et al*., 2006) and the present work with the *M. smegmatis* enzyme indicate that inhibition of DNA gyrase activity is a typical characteristic of at least one group of glutamate racemases. The interaction and inhibitory effect of MurI seems to be specific to gyrase and conserved across the species barrier. Because, MurI from distantly related organisms exhibit this bifunctional behavior, the bifunctionality does not seem to be a mere coincidence and may have some functional significance. It is suggested that the gyrase-inhibitory properties of glutamate racemases might have evolved in eubacteria to serve some important physiological purpose.

Table 1. Diameters of zone of inhibition for different dilutions of ciprofloxacin and mitomycin C on a lawn of *Mycobacterium smegmatis* strain harboring either the vector pJAM2 or pJAM2-*msmurI*

	pJAM2 vector (mm)	pJAM2-msmurI (mm)
Ciprofloxacin (μg)		
0.5	15 ± 0.6	10 ± 0.5
0.4	13 ± 0.5	9 ± 0.6
0.3	10 ± 0.6	5 ± 0.5
0.1	8 ± 0.5	3 ± 0.6
Mitomycin C (μg)		
20	12 ± 0.8	12 ± 0.5
10	9 ± 0.7	9 ± 0.4
5	7 ± 0.6	8 ± 0.1
2.5	5 ± 0.7	5 ± 0.6
1.25	4 ± 0.5	4 ± 0.2

Mycobacterium smegmatis cultures were grown in Middlebrook 7H9 broth till mid-logarithmic phase at 37 °C and then poured onto soft agar plates to form the lawn. Different dilutions of the drugs were spotted and then the plates were incubated for 3–4 days to get defined zones of inhibition.

DNA gyrase is a vital and indispensable enzyme in bacteria functioning in several DNA transaction processes. Among a large repertoire of the gyrase inhibitors, coumarins and quinolones have been studied extensively with respect to their mechanism of action (Lewis *et al*., 1996; Maxwell, 1999). Now, a newer group of inhibitors is emerging with the discovery of the chromosomally encoded inhibitors such as GyrI (Nakanishi *et al*., 1998; Chatterji & Nagaraja, 2001), MfpA (Montero *et al*., 2001; Hegde *et al*., 2005), Qnr (Martinez-Martinez *et al*., 2003; Tran *et al*., 2005; Arsene & Leclercq, 2007) and MurI (Ashiuchi *et al*., 2002, 2003; Sengupta *et al*., 2006). In spite of having no sequence or structural similarities, all these endogenous inhibitors share a common feature, as their mode of inhibition is to prevent binding of gyrase to DNA. Thus, none of these proteins are cytotoxic, as they do not arrest the enzymatic step of DNA gyrase. Cytotoxicity usually arises due to induction and accumulation of double-strand breaks in the genome following the trapping of gyrase in the form of enzyme–DNA covalent complexes, which form roadblocks to the cellular DNA transaction machineries.

In the present study, soluble mycobacterial MurI is purified by overexpressing it in *M. smegmatis* itself. Although it inhibits DNA gyrase activity *in vitro*, MurI protects *M. smegmatis* against the action of ciprofloxacin *in vivo*. These studies indicate that MurI may be a survival strategy used by the cell to safeguard the essential housekeeping enzyme, DNA gyrase from the gyrase-targeting poisons. Alternatively, MurI could bind transiently to DNA gyrase to sequester it away from DNA in situations wherein excessive supercoiling has to be modulated. Another plausible explanation is that MurI-mediated sequestration of gyrase serves as a 'check point' during the process of cell

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

division. In this model, the cell division and DNA replication would be coordinated by modulation of gyrase. An alarming increase in the emergence of multi-drug-resistant strains of *M. tuberculosis* and continuing high worldwide incidence of tuberculosis has motivated an increasingly active search for novel drug targets. Inhibition of the bacterial cell wall peptidoglycan biosynthesis pathway seems to be one of the attractive targets. MurI is already being considered as a target for designing new antibacterial drugs as it provides D-glutamate, a key building block for peptidoglycan in bacteria. Because MurI protects gyrase from the cytotoxic effect of quinolone drugs, blocking MurI activity along with antigyrase agents might be a more effective cell-killing strategy, providing a synergistic effect. The production and purification of mycobacterial MurI in large amounts in soluble form is a step forward to the design of high-throughput screening assays for testing potential MurI inhibitors with the aim of developing new antimycobacterial compounds.

Acknowledgements

The authors thank A. Maxwell for *E. coli* gyrase overexpression clones and acknowledge the Phosphorimager and proteomics facilities of the Institute supported by the Department of Biotechnology, Government of India. This work was funded by a Centre of Excellence tuberculosis research grant from the Department of Biotechnology, Government of India. S.S. is the recipient of a senior research fellowship from the Council of Scientific and Industrial Research, Government of India.

References

Arsene S & Leclercq R (2007) Role of a qnr-like gene in the intrinsic resistance of *Enterococcus faecalis* to fluoroquinolones. *Antimicrob Agents Chemother* **51**: 3254–3258.

Ashiuchi M, Kuwana E, Yamamoto T, Komatsu K, Soda K & Misono H (2002) Glutamate racemase is an endogenous DNA gyrase inhibitor. *J Biol Chem* **277**: 39070–39073.

Ashiuchi M, Kuwana E, Komatsu K, Soda K & Misono H (2003) Differences in effects on DNA gyrase activity between two glutamate racemases of *Bacillus subtilis*, the poly-gamma-glutamate synthesis-linking Glr enzyme and the YrpC (MurI) isozyme. *FEMS Microbiol Lett* **223**: 221–225.

Chatterji M & Nagaraja V (2001) GyrI: a counter-defensive strategy against proteinaceous inhibitors of DNA gyrase. *EMBO Rep* **3**: 261–267.

Chatterji M, Unniraman S, Mahadevan S & Nagaraja V (2001) Effect of different classes of inhibitors on DNA gyrase from *Mycobacterium smegmatis*. *J Antimicrob Chemother* **48**: 479–485.

de Dios A, Prieto L, Martin JA *et al.* (2002) 4-substituted D-Glutamic acid analogues: the first potent inhibitors of glutamate racemase (MurI) enzyme with antibacterial activity. *J Med Chem* **45**: 4559–4570.

Gallo KA & Knowles JR (1993) Purification, cloning and cofactor independence of glutamate racemase from *Lactobacillus*. *Biochemistry* **32**: 3981–3990.

Glavas S & Tanner ME (1997) The inhibition of glutamate racemase by D-*N*-hydroxyglutamate. *Bioorg Med Chem Lett* **7**: 2265–2270.

Glavas S & Tanner ME (2001) Active site residues of glutamate *racemase*. *Biochemistry* **40**: 6199–6204.

Gmunder H, Kuratli K & Keck W (1995) Effect of pyrimido [1, 6-α] benzimidazoles, quinolones and Ca^{2+} on the DNA gyrase-mediated cleavage reaction. *Antimicrob Agents Chemother* **39**: 163–169.

Hegde SS, Vetting MW, Roderick SL, Mitchenall LA, Maxwell A, Takiff HE & Blanchard JS (2005) A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. *Science* **308**: 1480–1483.

Hoskins JA, Norris FH, Rockey PK, Rosteck PR Jr, Skatrud PL, Treadway PJ, Young-Bellido M & Wu CE. Biosynthetic gene *mur*I from *Streptococcus pneumoniae* US Patent 5 948 645 September 7 1999.

Lewis RJ, Tsai FTF & Wigley DB (1996) Molecular mechanisms of drug inhibition of DNA gyrase. *BioEssays* **18**: 661–671.

Madhusudan K, Ramesh V & Nagaraja V (1994) Cloning and sequence analysis of DNA gyrase genes from *Mycobacterium tuberculosis*. *Curr Sci* **66**: 664–667.

Manjunatha UH, Dalal M, Chatterji M, Radha DR, Visweswariah SS & Nagaraja V (2002) Functional characterisation of mycobacterial DNA gyrase: an efficient decatenase. *Nucleic Acids Res* **30**: 2144–2153.

Martinez-Martinez L, Pascual A, Garcia I, Tran J & Jacoby GA (2003) Interaction of plasmid and host quinolone resistance. *J Antimicrob Chemother* **51**: 1037–1039.

Maxwell A (1999) DNA gyrase as a drug target. *Biochem Soc Trans* **27**: 48–53.

Maxwell A & Howells AJ (1999) Overexpression & purification of bacterial DNA gyrase. *Methods Mol Biol* **94**: 135–144.

Meroueh SO, Bencze KZ, Hesek D, Lee M, Fisher JF, Stemmler TL & Mobashery S (2006) Three-dimensional structure of the bacterial cell wall peptidoglycan. *Proc Natl Acad Sci USA* **103**: 4404–4409.

Montero C, Mateu G, Rodriguez R & Takiff H (2001) Intrinsic resistance of *Mycobacterium smegmatis* to fluoroquinolones may be influenced by new pentapeptide protein MfpA. *Antimicrob Agents Chemother* **45**: 3387–3392.

Nakanishi A, Oshida T, Matsushita T, Imajoh-Ohmi S & Ohnuki T (1998) Identification of DNA gyrase inhibitor (GyrI) in *Escherichia coli*. *J Biol Chem* **273**: 1933–1938.

Reck F, Marmor S, Fisher S & Wuonola MA (2001) Inhibitors of the bacterial cell wall biosynthesis enzyme, MurC. *Bioorg Med Chem Lett* **11**: 1451–1454.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sengupta S, Shah M & Nagaraja V (2006) Glutamate racemase from *Mycobacterium tuberculosis* inhibits DNA gyrase by affecting its DNA-binding. *Nucleic Acids Res* **34**: 5567–5576.

Tanner ME & Miao S (1994) The synthesis and stability of aziridinoglutamate; an irreversible inhibitor of glutamate racemase. *Tetrahedron Lett* **35**: 4073–4076.

Tanner ME, Vaganay S, van Heijenoort J & Blanot D (1996) Phosphinate inhibitors of the D-glutamic acid-adding enzyme of peptidoglycan biosynthesis. *J Org Chem* **61**: 1756–1760.

Tran JH, Jacoby GA & Hooper DC (2005) Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase. *Antimicrob Agents Chemother* **49**: 118–125.

Triccas A, Parish T, Britton WJ & Gicquel B (1998) An inducible expression system permitting the efficient purification of a recombinant antigen from *Mycobacterium smegmatis*. *FEMS Microbiol Lett* **167**: 151–156.

Unniraman S & Nagaraja V (1999) Regulation of DNA gyrase operon in *Mycobacterium smegmatis*: a distinct mechanism of relaxation stimulated transcription. *Genes Cells* **4**: 697–706.

Zeng B, Wong KK, Pompliano DL, Reddy S & Tanner ME (1998) A phosphinate inhibitor of the meso-diaminopimelic acid adding enzyme (MurE) of peptidoglycan biosynthesis. *J Org Chem* **63**: 10081–10086.

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved