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| Title | Quinolone resistance-associated amino acid substitutions affect enzymatic activity of Mycobacterium leprae DNA gyrase |
| Author(s) | Yamaguchi, Tomoyuki; Yokoyama, Kazumasa; Nakajima, Chie et al. |
| Citation | Bioscience, Biotechnology, and Biochemistry, 81(7), 1343-1347<br>https://doi.org/10.1080/09168451.2017.1314757 |
| Issue Date | 2017-04-18 |
| Doc URL | https://hdl.handle.net/2115/76493 |
| Rights | This is an Accepted Manuscript of an article published by Taylor & Francis in Bioscience, Biotechnology, and Biochemistry on 18 Apr 2017, available online: http://www.tandfonline.com/10.1080/09168451.2017.1314757 |
| Type | journal article |
| File Information | Main Text_accepte.pdf |

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# Quinolone resistance-associated amino acid substitutions affect enzymatic activity of *Mycobacterium leprae* DNA gyrase

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## Abstract

Quinolones are important antimicrobials for treatment of leprosy, a chronic infectious disease caused by *Mycobacterium leprae.* Although it is well known that mutations in DNA gyrase are responsible for quinolone resistance, the effect of those mutations on the enzymatic activity is yet to be studied in depth. Hence, we conducted *in vitro* assays to observe supercoiling reactions of wild type and mutated *M. leprae* DNA gyrases. DNA gyrase with amino acid substitution Ala91Val possessed the highest activity among the mutants. DNA gyrase with Gly89Cys showed the lowest level of activity despite being found in clinical strains, but supercoiled DNA like the wild type does if applied at a sufficient concentration. DNA gyrases with clinically unreported Asp95Gly and Asp95Asn supercoiled DNA in a different manner than the other DNA gyrases did. We speculate that this alteration may represent a reason this substitution is not found in clinical strains.

## Keywords

DNA gyrase, *Mycobacterium leprae,* quinolone resistance, amino acid substitution, enzymatic activity

# Introduction

*Mycobacterium leprae* is an unculturable bacterium that causes leprosy, also known as Hansen's disease. Although this chronic infectious disease had been considered to be incurable for a long time, it is now well accepted that this disease can be treated by chemotherapy [1]. Owing to a multidrug therapy introduced by the World Health Organization in the 1980s, world prevalence of leprosy has been dramatically reduced. Nonetheless, more than 210,000 new cases still occur every year mainly in Asian, Latin American, and African countries [2,3].

Quinolones, including fluoroquinolone, are recognized as an important antimicrobial class widely used for treatment of various bacterial infections including leprosy [4]. For example, quinolones are regularly used for treatment of single-skin lesion paucibacillary leprosy. Quinolones are also reportedly effective against multibacillary leprosy [5,6]. This antimicrobial class inhibits the activity of DNA gyrase, which is an essential enzyme for bacteria. DNA gyrase contributes to DNA transcription and replication by introducing negative supercoils into circular DNA to alleviate positive supercoils accumulated during those processes [7]. As quinolones interfere with DNA gyrase supercoiling activity by binding to the site where DNA gyrase cleaves double stranded DNA, amino acid substitutions at this quinolone-binding site can cause resistance to quinolones [8].

In clinical strains of *M. leprae*, two types of amino acid substitution in DNA gyrase subunits A (GyrA), glycine at position 89 to cysteine (Gly89Cys) and alanine at position 91 to valine (Ala91Val), have been found to be responsible for acquisition of quinolone resistance. However, it has been reported that the majority of quinolone-resistant *M. leprae* strains has Ala91Val substitution, and that Gly89Cys substitution can be found only in a very limited number of cases [9–11]. In addition to these amino acid substitutions found at clinical level, we previously demonstrated that experimentally-induced substitutions of aspartic acid at position 95 to glycine (Asp95Gly) and aspartic acid at position 95 to asparagine (Asp95Asn) also hinder the inhibitory activity of quinolones [12]. Moreover, although Asp95Gly and Asp95Asn have not been found in clinical *M. leprae* strains, their equivalent substitutions, aspartic acid at position 94 to glycine (Asp94Gly) and to asparagine

(Asp94Asn), are frequently reported in *M. tuberculosis* isolates [13–15].

As mentioned above, it is already clarified and proven that all four types of amino acid substitutions contribute to quinolone resistance. Nevertheless, the effect of these amino acid substitutions on *M. leprae* DNA gyrase activity itself, and the reason Asp95Gly has been unreported until now remain unclear. Hence, to further elucidate the enzymatic activity of wild type (WT) and mutant *M. leprae* DNA gyrases with Gly89Cys, Ala91Val, Asp95Gly and Asn95Asn, we conducted *in vitro* assays using recombinant DNA gyrase subunits.

## Materials and Methods

### Bacterial strains, expression plasmids and antimicrobial agents

Thai-53 strain of *M. leprae* [16], maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used to prepare *M. leprae* DNA. *Escherichia coli* TOP-10 (Thermo Fisher Scientific Inc.; Waltham, MA) was used for cloning. *E. coli* strains Rosetta-gami 2(DE3)pLysS and BL21(DE3)pLysS (Merck KGaA, Darmstadt, Germany) were used for protein expression. Plasmid vector pET-20b(+) (Merck KGaA) was used for the construction of expression plasmids. Relaxed pBR322 DNA (John Innes Enterprises Ltd.; Norwich, United Kingdom) was used for time course DNA supercoiling assays. Ampicillin (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used to select transformants.

### Construction of expression plasmids and purification of recombinant DNA gyrase subunits

DNA gyrase expression plasmids coding WT GyrA, GyrA with Gly89Cys (Gly89Cys-GyrA), GyrA with Ala91Val (Ala91Val-GyrA), GyrA with Asp95Gly (Asp95Gly-GyrA), GyrA with Asp95Asn (Asp95Asn-GyrA) and WT DNA gyrase subunit B (GyrB) were constructed as described in our previous studies [12,17].

Recombinant DNA gyrase subunits were expressed and purified as previously described [12,17–19]. The purified proteins were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Prestained Protein Marker, Broad Range (7-175 kDa) (New England Biolab; Hitchin, UK).

### Time course DNA supercoiling assay

Time course DNA supercoiling assays were carried out under the following conditions. Each assay was carried out in 30 μL of DNA gyrase reaction buffer (35 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1.8 mM spermidine, 24 mM KCl, 5 mM DTT, 0.36 mg/mL of BSA, 6.5% w/v glycerol, 1mM

ATP), with 2 nM relaxed pBR322 DNA, 8 nM GyrA (WT GyrA, Gly89Cys-GyrA, Ala91Val-GyrA, Asp95Gly-GyrA or Asn95Asn-GyrA) and 8 nM WT GyrB. The reactions were continued at 30 °C and stopped at 0, 10, 20, 40, 60, 90, 120, 240, 360 and 600 min by addition of 7.5 μL of 5× dye mix (5% SDS, 25% glycerol, 0.25 mg/mL of bromophenol blue). Next, 10 μL of each mixture was subjected to electrophoresis with Lambda DNA-HindIII Digest (New England Biolabs) on 1% agarose 1× TBE gels and stained with 1 μg/mL of ethidium bromide. To assess the activity of DNA gyrases, the amount of DNA supercoiled in the reactions was quantified with ImageJ (http://rsbweb.nih.gov/ij/) by comparing band brightness of the supercoiled DNA with that of the Lambda DNA-HindIII Digest. Each assay was conducted thrice to confirm its reproducibility.

For DNA gyrases with WT GyrA and Gly89Cys-GyrA, time course DNA supercoiling assay was also carried out using reaction mixtures of 4 nM relaxed PBR322 DNA, 8 nM GyrA and 8 nM WT GyrB (1:1 mixture of relaxed DNA and DNA gyrase), reaction mixtures of 4 nM DNA, 16 nM GyrA and 16 nM GyrB (1:2 mixture) and reaction mixtures of 4 nM DNA, 24 nM GyrA and 24 nM GyrB (1:3 mixture). The reactions were continued at 30°C and stopped at 0, 10, 20, 30, 40, 50, 60, 90, 120, 240, 360 and 600 min by addition of 7.5 μL of 5× dye mix. Gel electrophoresis and image analysis were conducted in a similar manner as described above. Each assay was conducted thrice to confirm its reproducibility.

# Results

## Alteration of DNA supercoiling activity by quinolone resistance-associated amino acid substitutions

Recombinant *M. leprae* GyrAs and GyrB were purified and subjected to time course DNA supercoiling assay. Each GyrA (WT, Gly89Cys-, Ala91Val-, Asp95Gly- or Asp95Asn-GyrA) was mixed with WT GyrB to form DNA gyrase and its supercoiling activity was observed for 600 min. Obtained electrophoretic images showed sequential changes of supercoiling by WT and mutant DNA gyrases (Fig. 1A). Then, reaction curves of DNA gyrases were obtained by analyzing the intensity of each band on the images at the position of the supercoiled form (Fig. 1B). WT DNA gyrase yielded the highest amount of supercoiled DNA. In comparison, the amounts of supercoiled DNA produced by DNA gyrases with Gly89Cys-, Ala91Val-, Asp95Gly- and Asp95Asn-GyrA at 600 minutes were 22.0%, 68.9%, 30.7% and 69.1%, respectively, of the amount produced by the WT (Table 1). Furthermore, in the assays using DNA gyrases with Asp95Gly- and Asp95Asn-GyrA, some part of the input relaxed DNA remained unsupercoiled at its initial position after the 10-hour reaction (Fig. 1A and 1C).

## Supplementation of reduced activity of Gly89Cys-substituted DNA gyrase by increasing the enzyme concentration

To further characterize the lowest level of supercoiling activity of DNA gyrase with Gly89Cys-GyrA, an additional time course assay was conducted by changing the molecular ratio of relaxed DNA and DNA gyrase from 1:1 to 1:3. Fig. 2 shows changes in the supercoiling reaction and the amount of supercoiled DNA when more DNA gyrase was introduced to the reaction mixture. WT DNA gyrase supercoiled relaxed DNA molecules more rapidly under a higher molecular ratio. DNA gyrase with Gly89Cys-GyrA also produced more supercoiled DNA when a larger amount of DNA gyrase subunits was added. Compared with the amount of supercoiled DNA produced under the 1:2

ratio, the amount of supercoiled DNA produced by DNA gyrase with Gly89Cys-GyrA under the 1:3 ratio was increased by 48.8%. The curve for the assay using Gly89Cys-GyrA under the 1:1 ratio could not be obtained because calculation of the amount of supercoiled DNA via image analysis was not possible.

## Discussion

In this study, focusing on the effects caused by quinolone resistant-conferring amino acid substitutions on the enzymatic activity of *M. leprae* DNA gyrase, we conducted time course DNA supercoiling assays to examine the supercoiling activity of WT and three mutant types of DNA gyrases. Our results clearly showed a particular effect of each amino acid substitution on the supercoiling activity of DNA gyrase (Fig. 1). Using time course assays, we showed that amino acid substitution Ala91Val causes the least effect on the supercoiling activity of *M. leprae* DNA gyrase. Even though our previous studies established that the level of quinolone-resistance conferred by this substitution was the lowest [12,17], considering the highest supercoiling activity among the tested mutant DNA gyrases, it seems reasonable that this amino acid substitution is selected by the majority of quinolone-resistant *M. leprae* strains.

Unlike DNA gyrase with Ala91Val amino acid substitution found at clinical level, DNA gyrase with Gly89Cys-GyrA showed the lowest supercoiling activity. Nonetheless, the fact that this amino acid substitution was found in clinical *M. leprae* strains is a strong evidence that DNA gyrase with Gly89Cys-GyrA does not totally lose enzymatic activity [9,10]. The electrophoretic image and its band intensities indicated that DNA gyrase with Gly89Cys-GyrA was gradually converting the entire set of input relaxed DNA molecules to the supercoiled form in a similar manner as DNA gyrases with WT and Ala91Val-GyrA did, even though the supercoiling was quite slow (Fig. 1). Therefore, it seems that the amount of DNA gyrase is important for the rate by which DNA gyrase with Gly89Cys-GyrA supercoils. To demonstrate that the DNA supercoiling rate can be supplemented by the molecular amount of DNA gyrase subunits, we conducted an additional assay of time course DNA supercoiling by changing the molecular ratio of circular DNA and DNA gyrase from 1:1 to 1:3 (from 1:2:2 to 1:6:6 as the ratio of circular DNA, GyrA and GyrB). In this assay, to clearly identify the difference between assay conditions, the amount of relaxed DNA was applied twice (4 nM) to assist the low supercoiling activity of DNA gyrase with Gly89Cys-GyrA. The results obtained from this assay clearly showed that the amount of supercoiled DNA produced by DNA gyrase with Gly89Cys-GyrA can be increased

when larger amounts of DNA gyrase subunits are added. In addition, both the electrophoretic images and the reaction curves showed that the supercoiling activity of DNA gyrase with Gly89Cys became more similar to that of WT, as a larger amount of enzymes was introduced (Fig. 2). These findings suggested that DNA gyrase with Gly89Cys can act similarly to that of WT as long as subunits are sufficiently expressed in the bacterial cells. However, it should be noted that the time course assays in this study had a limitation since they were not conducted with the same DNA and DNA gyrase concentrations as those found in actual bacterial cells. Therefore, to estimate and discuss the *in vivo* impact of Gly89Cys amino acid substitution on survival of *M. leprae*, further investigation to establish the number of molecules of DNA gyrase functioning inside individual bacilli of *M. leprae* is required.

Neither Asp95Gly nor Asp95Asn has been previously found in clinical strains of *M. leprae*, although their equivalent substitutions, Asp94Gly and Asp94Asn, are frequently reported in *M. tuberculosis*. We previously conducted *in vitro* assays using recombinant DNA gyrases with Asp95Gly and Asp95Asn, and demonstrated that these substitutions can also contribute to quinolone resistance [12,17]. It had been shown that the levels of quinolone resistance brought about by Asp95Gly and Asn95Asn are higher than that by Ala91Val, and the higher than that by Gly89Cys (Table 1). In this study, we revealed that those amino acid substitutions at position 95, especially Asp95Gly, largely decreased the supercoiling activity of *M. leprae* DNA gyrase. However, the supercoiling activities of *M. leprae* DNA gyrases with those amino acid substitutions were still higher than that by DNA gyrase with Gly89Cys-GyrA, which has been reported in clinical strains [9]. The supercoiling process between DNA gyrase with Asp95Gly- or Asp95Asn-GyrA and the other tested DNA gyrases was clearly different in the electrophoresis images (Fig. 1A). As DNA gyrase with Asp95Gly- or Asp95Asn-GyrA did not supercoil all the input relaxed DNA molecules, part of the relaxed DNA seemed to remain at its initial positions, whereas DNA gyrases found at clinical level, including WT, gradually supercoiled the entire set of input relaxed DNA molecules (Fig. 1A and 1C). It seems that the change in the supercoiling process reflected a defect in the function of *M. leprae* DNA gyrase caused by Asp95Gly

or Asp95Asn, which may be one of reasons for no previous report of Asp95Gly and Asp95Asn at clinical level, despite conferring high levels of quinolone resistance while keeping the supercoiling activity higher than Gly89Cys (Table 1). We speculate that DNA gyrase with Asp95Gly or Asp95Asn less frequently move out from the bound DNA molecule to another after giving a supercoil than the other DNA gyrases, and that the excessive persistence might be adverse to the function of DNA gyrase as an enzyme supporting DNA replication and transcription. Therefore, it was considered that quinolone resistance-conferring mutations in DNA gyrase are selected by multiplex factors, not only by the supercoiling activity or the level of quinolone resistance. To confirm these speculations, we plan further analysis to examine other DNA gyrase properties, including DNA-DNA gyrase binding affinity. In addition, more data of quinolone-resistant *M. leprae* strains should be accumulated since clinical reports of those strains are still too scarce to discuss and determine the possibility of Asp95Gly and Asp95Asn to occur in *M. leprae*.

In summary, we showed that amino acid substitutions that confer quinolone resistance to *M. leprae* DNA gyrase simultaneously cause diminution of enzymatic activity. Furthermore, it was demonstrated that amino acid substitutions Gly89Cys and Ala91Val reduced the enzymatic activity of DNA gyrase, but did not alter the innate supercoiling process of DNA gyrase as Asp95Gly and Asp95Asn did. From the results of this study, we conclude that the change in the supercoiling activity of DNA gyrase caused by the amino acid substitutions at position 95 in GyrA may represent one factor for the reason why these amino acid substitutions have never been previously reported in clinical strains of quinolone-resistant *M. leprae*.

## Acknowledgments

This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, for the Joint Research Program of the Research Center for Zoonosis Control, Hokkaido University to YS; in part by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 15J06295 to TY; in part by the Japan Initiative for Global Research Network on Infectious Diseases, Grant No. 15fm0108008h0001 from the Japan agency for Medical Research and Development (AMED) to YS; in part by the program on International Collaborative Research Program for Tackling the NTDs (Neglected Tropical Diseases) Challenges in African countries, Grant No. 15jm0510001h0002 from AMED; and in part by US-Japan Cooperative Medical Science Program, Grant No. 15jk0210005h0027 from AMED.

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# Table with captions

| Amino acid substitution in GyrA | Supercoiled DNA at 10 hours (% of WT) | Fold increase of $IC_{50}$ compared to WT[a] | Reported in clinical strains |
|---|---|---|---|
| WT | 100 | 1 | Yes |
| Gly89Cys | 22.0 | 10.7 [b] | Yes |
| Ala91Val | 68.9 | 5.8 [c] | Yes |
| Asp95Gly | 30.7 | 23.7 [c] | No |
| Asp95Asn | 69.1 | 38.6 [c] | No |

**Table 1.** Supercoiling activity and quinolone resistance of tested DNA gyrases

[a] Each value was calculated with $IC_{50}$s (concentrations that inhibit DNA supercoiling by 50%) of ofloxacin.

[b] Value calculated with data from Matrat et al. [20]

[c] Values referred from Yokoyama et al. [12]

## Figure captions

**Fig. 1.** Time-dependent DNA supercoiling by wild and mutant types of *M. leprae* DNA gyrases. (A) Relaxed pBR322 DNA (2 nM) mixed with DNA gyrase subunits GyrA (8 nM) and GyrB (8 nM), and ATP (1 mM) was incubated at 30°C. The supercoiling reaction was then stopped at each time point (as indicated) and examined by gel electrophoresis. R and SC denote the positions of the relaxed and supercoiling forms, respectively. (B) The amount of supercoiled DNA was calculated by analyzing the bands intensities at the position of the supercoiled form on electrophoresis images obtained from the time course DNA supercoiling assay. (C) Band intensities of electrophoretic images at the initial (0 min) and final (600 min) time points represent the amount of DNA molecules at the corresponding positions.

**Fig. 2.** Supercoiling activity of *M. leprae* DNA gyrase at various molecular concentrations. Supercoiling of (A) DNA gyrase with WT GyrA and (B) DNA gyrase with Gly89Cys-GyrA were examined under conditions of different subunit concentrations. Relaxed pBR322 DNA (4 nM), GyrA (8, 16 or 24 nM) and WT GyrB (8, 16 or 24 nM) were mixed with ATP (1 mM) and incubated at 30 °C. The reaction was stopped at each time point (as indicated) and then examined by gel electrophoresis. R and SC denote positions of the relaxed and supercoiling forms, respectively. (C) Band intensities at the position of the supercoiled form on the electrophoresis images were analyzed to calculate the amount of supercoiled DNA at the indicated time points.

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