Glutamate Racemase Is an Endogenous DNA Gyrase Inhibitor

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Running title:  A Novel Function of Glutamate Racemase
Almost all bacteria possess glutamate racemase to synthesize D-glutamate as an essential component of peptidoglycans in the cell walls. The enforced production of glutamate racemase, however, resulted in suppression of cell proliferation. In the *Escherichia coli* JM109/pGR3 clone, the overproducer of glutamate racemase, the copy number (*i.e.*, replication efficiency) of plasmid DNA declined dramatically, whereas the *E. coli* WM335 mutant that is defective in the gene of glutamate racemase showed little genetic competency. The comparatively low and high activities for DNA supercoiling were contained in the *E. coli* JM109/pGR3 and WM335 cells, respectively. Furthermore, we found that the DNA gyrase of *E. coli* was modulated by the glutamate racemase of *E. coli* in the presence of UDP-\(N\)-acetylmuramyl-L-alanine, which is a peptidoglycan precursor and functions as an absolute activator for the racemase. This is the first finding of the enzyme protein participating in both D-amino acid metabolism and DNA processing.
Bacterial cell walls contain D-amino acids as essential components of peptidoglycans (alternatively, mureins). D-Glutamate is introduced into peptidoglycan through its addition to UDP-\(N\)-acetylmuramyl-L-alanine (UDP-MurNAc-L-Ala), a peptidoglycan precursor, by UDP-MurNAc-L-Ala: D-glutamate ligase (EC 6.3.2.9). Glutamate racemase (EC 5.1.1.3) catalyzes the racemization of glutamate (1). The genes encoding glutamate racemase are ubiquitously inherited in bacteria, and the *Escherichia coli* WM335 mutant, in which the enzyme gene was disrupted, required D-glutamate for growth (2). These findings indicate that glutamate racemase provides D-glutamate for peptidoglycan synthesis. Glutamate racemase and its gene, hereafter, are designated MurI and *murI*, respectively.

The activity of glutamate racemase, however, cannot be usually detected in the cells of bacteria, except a part of lactobacilli and bacilli (3). Doublet et al. (4) showed that MurI of *E. coli* catalyzed the glutamate racemization only in the presence of UDP-MurNAc-L-Ala and concluded that this peptidoglycan precursor is an absolute activator of the MurI enzyme. On the other hand, the fact that no ribosome-binding sequence is found at the upstreams of the open reading frames (ORFs) in the *murI*s of various bacteria and the initial codon of the ORFs often includes the substitution of the usual ATG for the unusual TTG or GTG (5, 6) suggests that the *murI* gene is translated on a specific mechanism that is still unidentified. It is generally assumed that MurI is strictly controlled
so as to operate only during peptidoglycan synthesis (eventually during cell division). In E. coli clones, the enforced production of MurIs resulted in characteristic changes, such as aberration in nucleoid separation (7) and suppression of cell proliferation (6, 7), indicating that the attenuation of murI-gene expression and the regulation of MurI production are physiologically significant. In the absence of D-glutamate, the cells of the E. coli WM335 mutant formed filament elongates (2), as did the mutants defective in the genes involved in cell division. These observations imply that MurI plays a role in cell homeostasis other than in the D-glutamate supply. However, there have been surprisingly few examples focusing on the multi-functionality of MurI until now. In this study, the in vivo and in vitro effects of MurI on some DNA processing in E. coli were investigated. Here we report the novel function of glutamate racemase, i.e., the modulation of DNA gyrase activity.
EXPERIMENTAL PROCEDURES

**Materials** — Supercoiled pBR322, calf thymus DNA topoisomerase I, and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from TaKaRa Shuzo, Japan. Relaxed pBR322 was prepared from supercoiled pBR322 with the DNA topoisomerase I by the method of Ferro et al. (8).

UDP-MurNAc-L-Ala was prepared from the cell extract of *E. coli* WM335 according to the method previously (6). A novobiocin-Sepharose resin was prepared by the method of Nakanishi et al. (9). All other chemicals were of analytical grade.

**Bacteria** — *E. coli* JM109 was purchased from TaKaRa Shuzo, Japan. *E. coli* WM335 mutant was a kind gift from Prof. Dr. W. Messer of the Max-Plank Institute for Molecular Genetics, Germany.

**Vector Plasmids** — Vector plasmids pKK223-3 and pTrc99A, both of which contain an ampicillin (Amp)-resistance gene, were obtained from Pharmacia Biotech., Sweden, and pACYC184 that carries a tetracycline (Tet)-resistance gene was from Nippon Gene, Japan. Both plasmids pGR2 and pGR3 were constructed according to the strategy described previously (5) and used for analysis of the function of glutamate racemase. For the production of *E. coli* DNA gyrase (GyrAB), plasmid pGYRA, a pTrc99A having the gyrA gene, and plasmid pGYRB, a pTrc99A having the gyrB gene, were designed by the method of Mizuuchi et al. (10). To obtain *E. coli* DNA topoisomerase IV (ParCE), plasmid pPARC, a pTrc99A having the perC gene, and plasmid pPARE,
a pTrc99A having the parE gene, were prepared by the method of Kato et al. (11).

**Transformation** —— *E. coli* cells were transformed with the vector plasmid DNA by the CaCl$_2$ method (12) or the TSS method (13) and grown on the LB media containing appropriate antibiotics (1, 12).

**Measurement of Plasmid Copy Number** —— Plasmid copy numbers were determined densitometrically with a Digital Science EDAS 120 LE system (Gibco BRL, Grand Island) by the method of Projan et al. (14).

**Enzyme Assays** —— Glutamate racemase was assayed by the method described previously (3). One unit of MurI was defined as the amount of enzyme that catalyzed the formation of 1 µmol of L-glutamate per min.

DNA gyrase was assayed as follows. The reaction mixture (20 µl) comprised 2 µmol of Tris-HCl (pH 8.0), 1.4 µmol of KCl, 0.2 µmol of MgCl$_2$, 0.1 µmol of ATP, 0.1 µmol of spermidine hydrochloride, 0.1 µmol of dithiothreitol (DTT), 2 nmol of EDTA, 0.2 mg of glycerol, 0.5 µg of relaxed pBR322, and enzyme. The enzyme was replaced with water in a blank. The reaction was essentially performed at 37°C for 1 h and terminated by the phenol-chloroform extraction (12). The reaction mixture was electrophoresed in 1% (w/v) agarose gel (14 x 14 cm) submerged in TPE buffer (90 mM Tris-phosphate, 2 mM EDTA, pH 8.0) at 4 V/cm at 25°C for 2.5 h. Gel was stained with ethidium bromide (1 µg/ml). The activity was estimated from both an increase in the density of bands corresponding to the supercoiled DNA thus formed (10) and a decrease in that of the relaxed DNA substrate, which
were quantitated with the Digital Science EDAS 120 LE system. One unit of GyrAB was defined as the amount of enzyme that converted a half of relaxed pBR322 into the supercoils per 30 min.

DNA topoisomerase I (TopA) and IV (ParCE) were assayed in the absence (15) and the presence of ATP (11), respectively. One unit of both topoisomerases was defined as the amount of enzyme required to fully relax 0.5 µg of supercoiled pBR322 per hour.

Enzymes — *E. coli* MurI was purified by the method described previously (1). The specific activity was 9.6 U mg⁻¹. Protein concentrations were determined spectrophotometrically using its theoretical molar extinction (ε₂₇₈ = 22,280 M⁻¹ cm⁻¹) (1, 4).

Since overproduction of DNA gyrase into an active form, i.e., a GyrA₂B₂ heterotetramer (molecular mass, 373,767 Da), results in strong inhibition of cell growth in bacteria, *E. coli* GyrAB was prepared by reconstitution of the A subunit (GyrA; 96,975 Da) and the B subunit (GyrB; 89,893 Da) (10). Cell lyses with lysozyme and Brij-58, removal of the DNA extracted with streptomycin, and precipitation of proteins with ammonium sulfate were conducted according to the method of Nakanishi *et al.* (9). The GyrA preparation from *E. coli* JM109/pGYRA cells (110 mg of protein) was applied to a Bio-Rad Bio-Logic FPLC system equipped with a Bio-Scale DEAE anion-exchange column (volume, 20 ml; Bio-Rad) (3); the fraction eluted with 0.4 M NaCl contained only the GyrA protein. The GyrB preparation from *E. coli* JM109/pGYRB cells (88 mg of protein) was subjected to affinity
chromatography on a novobiocin-Sepharose column (1.5 × 7 cm; volume, 12.5 ml) (9); the fraction eluted with 2 M KCl plus 5 M urea contained only the GyrB protein. The enzyme was reconstituted by incubation of the purified GyrA and GyrB at an equimolar ratio at 25°C for 30 min in the presence of 0.1 mM ATP. The specific activity was 3.8 × 10^4 U mg⁻¹. Protein concentrations were determined using its theoretical molar extinction (ε<sub>278</sub> = 248,200 M⁻¹ cm⁻¹) (10).

*E. coli* ParCE was purified by the method of Kato et al. (11). The specific activity was 3.3 × 10³ U mg⁻¹. Protein concentrations were determined using its theoretical molar extinction (ε<sub>278</sub> = 229,920 M⁻¹ cm⁻¹) (11).
RESULTS

Effects on Plasmid Copy Number — To examine whether the production of MurI affects DNA processing in *E. coli*, we first assayed the plasmid copy numbers in several *E. coli* clones. In this experiment, both plasmid pGR2, a pKK223-3 having the *murI* gene isolated from the *E. coli* chromosome, and plasmid pGR3, a pKK223-3 having the designed *murI* gene in which a typical ribosome-binding sequence was introduced (5), were used. The plasmid pKK223-3, the multiplication of which is entirely dependent on the replication machineries of the host cells, *i.e.*, the theta mechanism (16), was used as a control (usually over 20 copies). As shown in Fig. 1, the enforced production of MurI by the use of the plasmid pGR3 resulted in a dramatic decrease in the copy numbers in both *E. coli* JM109 and WM335 clone cells (one to several copies). The result also showed that a decreased plasmid copy number in the *E. coli* WM335 mutant was restored by complementation of the native *murI* gene with the plasmid pGR2. A decline in negative superhelicity of the plasmid in the MurI overproducer, *E. coli* JM109/pGR3, but not of those in *E. coli* JM109/pKK223-3 and JM109/pGR2 was observed by the use of chloroquine (data not shown), and this was consistent with what Balikó and Venetianer (7) had proposed. It thus seems likely that the replication of ColE1-type plasmids such as pKK223-3 is difficult to complete properly in *E. coli* cells exhibiting aberration of the production of MurI due to deficient in the controls of DNA superhelical density (11). MurI would also influence the
replication of chromosome, since the overexpression and disruption of the \textit{murI} gene allowed the appearance of abnormal cell growth in \textit{E. coli} (2, 6, 7).

\textbf{Effects on Genetic Competence} —— While examining characteristics of the \textit{E. coli} WM335 mutant, we found that its genetic competence declined dramatically compared with that of \textit{E. coli} JM109, which has an intact \textit{murI} gene, and, further, that such a decline in genetic competence of the mutant was recovered by the complementation of the \textit{murI} gene with the plasmid pGR2 (Table 1). Chandler and Smith (17) recently reported that, in bacteria, TopA catalyzing the reverse of the DNA gyrase reaction was essential for the development of genetic competence. The relationship between the development of the genetic competence and the intracellular activity for DNA supercoiling was demonstrated. Our results indicated that MurI affects the intracellular activity for DNA supercoiling.

\textbf{Effects on DNA Supercoiling Activities} —— The intracellular activity for DNA supercoiling was practically determined in the balance of the activities of TopA and GyrAB (18), and net DNA supercoiling activity of bacterial cells can be determined by using the DNA gyrase assay system because the cell extracts essentially contain both the activities of GyrAB, which introduces negative supercoils into DNAs, and of TopA, which unwinds the supercoiled DNAs. In this study, comparatively low and high DNA supercoiling activities were observed in the cells of \textit{E. coli} JM109/pGR3 and WM335, respectively (Fig. 2). The supercoiling activity of \textit{E. coli} JM109/pGR3 cells was a tenth of that of \textit{E. coli} JM109/pKK223-3 cells; the intracellular activities of \textit{E. coli} WM335 and \textit{E. coli}
coli WM335/pGR3 were 2-fold higher and 4-fold less than that of *E. coli* WM335/pGR2, respectively. In contrast, there was apparently little difference in the activities of TopA among the *E. coli* clones used: JM109/pKK223-3, 31 ± 7 (U mg⁻¹); JM109/pGR2, 34 ± 12; JM109/pGR3, 39 ± 14; WM335/pKK223-3, 28 ± 13; WM335/pGR2, 30 ± 5; WM335/pGR3, 33 ± 6. It was thus suggested that MurI affects the intracellular activity of GyrAB in *E. coli*.

**Inhibition of DNA Gyrase by Glutamate Racemase** —— To clarify if MurI modulates GyrAB, we followed a change in the apparent activity of GyrAB in the coexistence of various concentrations of MurI. BSA, instead of *E. coli* MurI, was added to the DNA gyrase assay system as a negative control (Fig. 3, open diamonds). As shown in Fig. 3A, GyrAB was inhibited by *E. coli* MurI in the presence of UDP-MurNAc-L-Ala (closed circles) but not both enantiomers of glutamate (open triangles and open squares). Figure 3A also reveals that GyrAB probably forms a 1:1 molar stoichiometric inactive complex with MurI activated by UDP-MurNAc-L-Ala. The apparent inhibition constant $K_{i(app)}$ of MurI against GyrAB was estimated to be 6.8 nM (Fig. 3B). On the other hand, *E. coli* MurI (0.5 nM) kept the activity of glutamate racemase even in the presence of 250-fold molar excess of GyrAB.

**Effect of Glutamate Racemase on the Activity of DNA Topoisomerase IV** —— ParCE, a GyrAB paralogue, participates in the chromosome partitioning (11) that occurs after the DNA replication in cell division. The enzyme (20 nM), however, remained active when coexisting with abundant MurI (125 nM). These suggest that DNA gyrase is the target of glutamate racemase in *E. coli*.
DISCUSSION

DNA gyrase (alternatively, bacterial DNA topoisomerase II) is a pivotal enzyme for various types of DNA processing, including DNA replication and gene expression, and the strict modulation of the intracellular activity is indispensable for proper cell division (10). The first endogenous DNA gyrase modulator, designated as GyrI (9), was identified from *E. coli*; it was identical to the SbmC protein, which had been previously characterized as a member of the DNA repair system. Because glutamate racemase, however, has been classified into the group of D-amino acid-metabolic enzymes but not into that of DNA-processing proteins, such as the GyrI protein, and does not have any consensus sequences that can be observed in the DNA-binding motifs (3, 5, 6), the discovery of the novel function of glutamate racemase as the endogenous inhibitor for DNA gyrase was quite unexpected. Nevertheless, the existence of such multifunctional glutamate racemases would be advantageous in the peculiar processes seen only during bacterial cell division, in which the synthesis of the peptidoglycan molecules (as an essential constituent of the septum and cell walls) follows DNA processing (*e.g.*, the decatenation of daughter DNAs catalyzed by DNA gyrase) (11), for example.

In bacterial glutamate racemases, *E. coli* MurI is functionally particular: it is led into an active form by UDP-MurNAc-L-Ala (4). *E. coli* MurI thus operates only during the peptidoglycan synthesis and changes again into an inactive form with exhaustion of UDP-MurNAc-L-Ala after peptidoglycan
synthesis. As shown in Fig. 3A, UDP-MurNAc-L-Ala was essential for the inhibition of GyrAB by *E. coli* MurI, suggesting that the function of DNA gyrase is suppressed by glutamate racemase at the early stage of the septation in *E. coli* cells. It seems likely that this step is important to avoid the appearances of abnormal DNA replication and decatenation due to excessive DNA gyrase activity. We have identified the glutamate racemase isozyme of *Bacillus subtilis*, YrpC (lately *B. subtilis* MurI), which was not modulated by UDP-MurNAc-L-Ala (6). Unlike *E. coli* MurI, *B. subtilis* MurI, whether coexisting with UDP-MurNAc-L-Ala or not, inhibited DNA gyrase². Although Doublet *et al.* (4) previously mentioned a toxicity of D-glutamate produced by glutamate racemase on the metabolism and growth of bacterial cells, our observations show that an active form of glutamate racemase itself but not its reaction product, *i.e.*, D-glutamate, is required for the inhibition of DNA gyrase, a most crucial enzyme in bacterial cell homeostasis.

Unlike usual amino acid racemases, glutamate racemase contains no coenzymes such as PLP (3). There is no sequence homology between glutamate racemase and usual amino acid racemases (6). The catalytic efficiency of glutamate racemase is exceedingly low compared to those of PLP-dependent amino acid racemases (5). Interestingly, the orthologues of glutamate racemase have been found from various organisms, including peptidoglycan-less organisms such as archaea, plants, and humans (19). Most of these proteins, however, have not been characterized yet because of their loss of function as glutamate racemase. It was recently reported that the glutamate
racemase orthologue-encoding gene was expressed to excess in human carcinoma (19), in which abnormal DNA replication frequently took place during cell division. On the other hand, among other PLP-independent amino acid racemases, the proline racemase from *Trypanosoma cruzi* was found to exhibit the function as an immune B-cell mitogen (20). Some PLP-independent amino acid racemases including glutamate racemase have possibly evolved from ancient proteins that had not been originally involved in amino acid metabolism. It remains to be investigated whether glutamate racemase-like proteins substantially modulate DNA topoisomerases, as their detailed structural analyses could provide insights into the molecular evolution of D-amino acid-metabolic enzymes, deepen the understanding of the mechanism of cell division, and result in the design of novel pharmaceuticals.


16
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1 Abbreviations used are: UDP-MurNAc-L-Ala, UDP-N-acetylmuramyl-L-alanine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PLP, pyridoxal 5’-phosphate; PEG, polyethylene glycol.

2 Ashiuchi, M., Kuwana, E., Komatsu, K., Soda, K., and Misono, H., manuscript in preparation.
FIG. 1. **Effects of the enforced production of glutamate racemase on the plasmid copy number in *E. coli* cells.**  
*Panel A*, the plasmid copy numbers of each *E. coli* JM109 clone harboring pKK223-3 (empty vector), pGR2 (vector carrying the *murI* gene from *E. coli*), or pGR3 (vector for enforced expression of the *murI* gene (5)). *Panel B*, the plasmid copy numbers of each *E. coli* WM335 clone harboring pKK223-3, pGR2, or pGR3. These *E. coli* clones were cultivated at 37°C in 10 ml of the D-glutamate-LB broth (2) containing Amp (50 μg/ml) and IPTG (1 mM) until the turbidity at 600 nm of the culture broth reached 2.1. The whole DNA was isolated by the method of Saito and Miura (21) and the plasmid DNA was prepared by the alkali-SDS method (12). The plasmid copy number was defined to be one when 1 ng of the plasmid DNA was contained in 1 μg of the whole DNA on the basis of the difference in molecular sizes between the plasmid DNA used and the chromosomal DNA of *E. coli*. Data are representative as means ± s.e.m. of eight independent experiments.

FIG. 2. **Effects of the enforced production of glutamate racemase on the DNA supercoiling activity in *E. coli* cells.** *Panel A*, the DNA supercoiling activity of each *E. coli* JM109 clone harboring pKK223-3, pGR2, or pGR3. *Panel B*, the DNA supercoiling activity of each *E. coli* WM335 clone harboring pKK223-3, pGR2, or pGR3. These *E. coli* clones were cultivated at 37°C in 100 ml of the D-glutamate-LB broth containing Amp (50 μg/ml) and IPTG (1 mM) until the
turbidity at 600 nm of the culture broth reached 2.1. Cell extracts (0.5 ml) were prepared by the use of lysozyme (0.3%), Brig-52 (1%), and streptomycin (2%) and dialyzed against TED buffer (pH 7.5) containing 1% glycerol (9). The dialyzed sample (10 µg of protein) was added to the DNA gyrase assay mixture (see the “EXPERIMENTAL PROCEDURES”). Data are representative as means ± s.e.m. of four independent experiments.

FIG. 3. Dose-dependent inhibition of DNA gyrase by glutamate racemase. A, titration of DNA gyrase with glutamate racemase. E. coli GyrAB (20 nM) was incubated at 37°C for 10 min in the DNA gyrase assay mixture (20 µl) containing various concentrations (0 to 50 nM) of E. coli MurI (open circles), E. coli MurI plus UDP-MurNAc-L-Ala (10 µM) (closed circles), E. coli MurI plus D-glutamate (50 mM) (open triangles), E. coli MurI plus L-glutamate (50 mM) (open squares), or BSA plus UDP-MurNAc-L-Ala (open diamonds). B, determination of the $K_{i(app)}$ value of glutamate racemase against DNA gyrase. A low concentration of E. coli GyrAB (0.5 nM) was incubated at 37°C for 2 h with various concentrations (0 to 125 nM) of E. coli MurI and UDP-MurNAc-L-Ala in the same assay mixture as (A). During the DNA gyrase reaction, both the supercoiled DNA products thus formed and the relaxed DNA substrate thus exhausted were determined densitometrically (see the “EXPERIMENTAL PROCEDURES”).
TABLE I

*Competency of E. coli WM335 mutant defective in the glutamate racemase gene*

*E. coli* JM109 transformants with pKK223-3 or pACYC184, *E. coli* WM335 transformants with pKK223-3 or pACYC184, and both *E. coli* JM109/pGR2 and WM335/pGR2 transformants with pACYC184 were grown at 37°C for 24 hr on an LB plate (12) containing Amp (50 µg/ml) or Tet (12.5 µg/ml), a D-glutamate-LB plate (2) containing Amp or Tet, and an LB plate containing Amp, Tet, and 0.1 mM IPTG, respectively. Competency of each *E. coli* recipient was assessed on the basis of the number of colonies being formed. Data are representative as an average of eight independent experiments.

<table>
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<th><em>E. coli</em> recipients</th>
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<tr>
<td></td>
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<td>0.8 x 10\textsuperscript{2}</td>
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\textsuperscript{a} Fifteen ng of the DNA was used for the transformation experiment.

\textsuperscript{b} An *E. coli* transformation method with CaCl\textsubscript{2}; Sambrook *et al.* 1989 (12).

\textsuperscript{c} An *E. coli* transformation method with PEG; Chung *et al.* 1989 (13).
Figure 2
Figure 3

(A) Residual DNA gyrase activity (%) as a function of [E. coli MurI] (nM).

(B) Inhibition of DNA gyrase (%) as a function of [E. coli MurI] (nM). The apparent dissociation constant ($K_{i(app)}$) is 6.8 nM.
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