Identification of DNA Gyrase Inhibitor (GyrI) in *Escherichia coli*

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DNA gyrase is an essential enzyme in DNA replication in *Escherichia coli*. It mediates the introduction of negative supercoils near oriC, removal of positive supercoils ahead of the growing DNA fork, and separation of the two daughter duplexes. In the course of purifying DNA gyrase from *E. coli* KL16, we found an 18-kDa protein that inhibited the supercoiling activity of DNA gyrase, and we coined it DNA gyrase inhibitory protein (GyrI). Its NH₂-terminal amino acid sequence of 16 residues was determined to be identical to that of a putative gene product (a polypeptide of 157 amino acids) encoded by *geeB* (EMBL accession no. U00009) and *sbmC* (Baquero, M. R., Bouzon, M., Varea, J., and Moreno, F. (1995) *Mol. Microbiol.* 18, 301–311) of *E. coli*. Assuming the identity of the gene (gyrI) encoding GyrI with the previously reported genes *geeB* and *sbmC*, we cloned the gene after amplification by polymerase chain reaction and purified the 18-kDa protein from an *E. coli* strain overexpressing it. The purified 18-kDa protein was confirmed to inhibit the supercoiling activity of DNA gyrase *in vitro*. *In vivo*, both overexpression and antisense expression of the *gyrI* gene induced filamentous growth of cells and suppressed cell proliferation. GyrI protein is the first identified chromosomally nucleoid-encoded regulatory factor of DNA gyrase in *E. coli*.

DNA gyrase, a type II topoisomerase in *Escherichia coli*, has the ability to cut a double-stranded DNA, pass an uncut portion of the duplex between the cut ends, and reseal the cut. It can introduce negative supercoils into covalently closed circular DNA and cause catenation and decatenation of two different DNA duplexes, *in vitro* (1). It has been established that the enzyme is essential for chromosomal replication *in vivo* (2). Moreover, there have been reports on the involvement of DNA gyrase in transcription from certain operons, DNA repair, and recombination in *E. coli* (2).

DNA gyrase is composed of two subunits, A (GyrA) and B (GyrB), which are assembled in A₂B₂ complexes, the active form (3–5). The active complex has been purified from *E. coli* (6) and reconstituted from the purified GyrA and GyrB (7–9). GyrA has an active center for the reactions of introducing and resealing the cuts of double-stranded DNA, whereas GyrB powers the reaction by catalyzing ATP hydrolysis.

DNA gyrase is a target of two distinct classes of inhibitors, coumarins (10, 11) and quinolones (10, 12). Coumarins bind to GyrB and are competitive inhibitors with respect to ATP (11). In contrast, quinolones bind DNA gyrase when the enzyme is complexed with DNA and trap the enzyme in an abortive ternary complex, which, upon treatment with a denaturant, releases cleaved DNA with GyrA covalently attached to the 5'-phosphoryl ends generated at the cut site.

There have been several reports on regulating DNA gyrase activity in *E. coli*. LetD (13) encoded on F factor inhibits DNA gyrase activity via the induction of synthesis of heat shock proteins (14). Another regulatory factor, cyclic AMP (cAMP) receptor (15), participates in regulation of the growth phase-dependent transcription of *gyrA* (15).

In this study, we discovered an 18-kDa protein, termed DNA gyrase inhibitory protein (GyrI), which could inhibit the supercoiling activity of DNA gyrase in *E. coli* KL16. We describe here the purification and characterization of GyrI and phenotypic analyses of recombinant strains overproducing GyrI or expressing antisense *gyrI* gene to decipher importance of GyrI in regulation of DNA gyrase activity *in vivo*.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** *E. coli* strains used in this study were JM109, G724 (F' λ lacI* lacPL8 ampC*:Pcr I merA merB INV(rnt-rrnE)), and KL16 (HfrKL16-P045[thyA-serA]-serB-thrI-relAI (λ*)). A plasmid pGLacZ7 (16) was used for construction of a reporter plasmid to monitor the strength of the gyrI promoter.

pBR322 and pLEX were purchased from Takara and Invitrogen, respectively.

**Assay of DNA Supercoiling Activity of DNA Gyrase—** The supercoiling activity was measured by the method of Sato et al. (17). One unit of enzyme activity was defined as the amount that brought 50% of relaxed pBR322 to the supercoiled position in agarose gel electrophoresis as described by Gellert et al. (18). The reaction mixture (10 μl) contained 25 mM Tris-HCl (pH 8.0), 67 mM KCl, 5 mM MgCl₂, 1.25 mM spermidine hydrochloride, 1.7 mM ATP, 20 μg of *E. coli* tRNA/ml, and 0.15 μg of relaxed pBR322 DNA. pBR322 was relaxed by using topoisomerase I as described by Takahata and Nishino (19). After the addition of 1 unit (0.23 μg of protein) of the holoenzyme reconstituted from the purified subunits, the reaction mixture was incubated at 37 °C for 2 h. The reaction was stopped by supplementation with 20 μg/ml proteinase K, and the mixture was subjected to 1% agarose gel electrophoresis. The gel was stained with ethidium bromide (0.5 μg/ml) and photographed.

The supercoiling activity was calculated from the density of the band of supercoiled DNA, which was quantitated using a densitometer with the negatives.

**Purification of GyrA and GyrB Subunits—** Subunits A (GyrA) and B (GyrB) of DNA gyrase were purified from *E. coli* KL16 (20). Cultivation of the bacteria, preparation of bacterial lysate, and removal of DNA by successive streptomycin and ammonium sulfate precipitation were conducted according to the method of Aoyama and co-workers (17, 20). The solution obtained after ammonium sulfate precipitation was loaded onto a Novosibirin-Sepharose column previously equilibrated with TED buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM diithiothreitol). The proteins were eluted stepwise by 0.2 M KCl, 2 M KCl, 5 M urea, and 2 M KCl plus 5 M urea in TED buffer. The holoenzyme and GyrA and GyrB subunits were eluted by TED buffer containing 5 M urea, 2 M KCl, and 2 M KCl plus 5 M urea, respectively. The fractions containing GyrA were loaded onto a column of heparin-Sepharose CL-6B equilibrated...
with TED buffer. The column was washed with TED buffer containing 100 mM KCl, and the activity was eluted with TED buffer containing 2 mM KCl. The fractions containing GyrB were purified further by chromatography on Novobiocin-Sepharose column again. The sample was applied to the column, washed with TED buffer containing 100 mM KCl, and then eluted with a linear gradient of NaCl (0.025–0.7 M) in 140 ml total of TED buffer. An aliquot of each fraction was analyzed using SDS-PAGE, and the fractions containing the 18-kDa protein were pooled. The pooled fractions (4 ml) were applied to a TSKgel TOYOPAQUE HR-55 column (TOSO, 0.4 cm² × 18 cm) and chromatographed with TED buffer. The fractions containing the 18-kDa protein were quickly frozen in small aliquots using liquid nitrogen.

Analysis of Protein—SDS-PAGE (12%) was carried out according to the method of Laemmli (23). Electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane (Millipore Corp.) using the method of Laemmli (23). Electrophoresed proteins were transferred onto a polyvinylidene difluoride membrane (Millipore Corp.) using a Transblot System. After blotting, the polyvinylidene difluoride membrane was sliced into 1-mm strips and wrapped in Saran Wrap. The tips of the strips were cut off with a razor blade, and 0.1 ml of the solution was loaded into each well of a slab gel. The gel was then placed in a gel tray and covered with a cover sheet. The gel was electrophoresed for 2 h at 80 V. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 for 30 min, and then destained with 10% acetic acid and 40% methanol for 2 h. The gel was photographed under an ultraviolet transilluminator.

Preparation of Antibody to GyrI—Antibody to the purified Gyr protein was raised in rabbits by the method of Liu et al. (29). The immunogen (0.1 mg of protein) was injected subcutaneously to rabbits with 0.025 ml of adjuvant (CFA). Immunization was performed after 3 weeks in the same manner as the first one, and thereafter immunizations were carried out at 7-day intervals. The antibody titer in antiserum was monitored by dot-blot assay. When the antibody titer increased sufficiently, the rabbit was bled from the ear artery (30–50 ml). Antibody was purified from the antiserum by using E-Z-SEP Polyclonal kit (Pharmacia Biotech Inc.) according to the protocol recommended by the manufacturer, to give an IgG fraction of the antiserum. The purified antibody was aliquoted and stored at −20°C.

Immunological Detection of GyrI— Dot-blot assay was carried out as follows. Protein and bovine serum albumin were mixed in phosphate-buffered saline at final concentrations of 0.1 mg/ml. Each mixture of protein and albumin was incubated with TBS containing 0.05% of anti-rabbit IgG/ml of TBS containing 0.05% of anti-rabbit IgG/ml. The antibody was visualized by color development with the peroxidase substrates 3,3′-diaminobenzidine and hydrogen peroxide. Western blotting was performed as described above. The protein band of GyrI was identified by immunological staining of the protein-blotted membrane as in the dot-blot assay. Microscopic Observation of Nucleoids—Nucleoids of E. coli cells were stained with ethidium bromide (33). The bacterial cells were washed with M9 medium (42 mM Na₂HPO₄, 2 mM KH₂PO₄, 8.5 mM NaCl, and 18 mM NH₄Cl (pH 7.4)), suspended in a solution containing 10 mM Tris-Cl (pH 7.4), 5 mM EDTA, and 0.05% 2-mercaptoethanol, 0.05% chloramphenicol, and 0.8% ethanol, and then mixed with an equal volume of 0.01% ethidium bromide solution. The nucleoids were observed and photographed with an incident fluorescence microscope equipped with phase-contrast optics (Nikon HFX).

The abbreviations used are: PAG, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid; bp, base pair; CPU, colony-forming units.
RESULTS

Purification of 18-kDa Protein—DNA gyrase was partially purified by using affinity chromatography on a novobiocin-Sepharose column (Fig. 1). The holoenzyme of DNA gyrase consisting of both GyrA and GyrB subunits was eluted by 2 M KCl. GyrB subunit was found in the fractions of 5 M urea, whereas addition of fraction consisting of both GyrA and GyrB subunits was eluted by 5 M Sepharose column (Fig. 1). The holoenzyme of DNA gyrase purified by using affinity chromatography on a novobiocin-Sepharose column was eluted by 2 M KCl. GyrB subunit was found in the fractions of 5 M urea. The activity of the GyrA subunit was found in the fractions (Fig. 1). DNA supercoiling activity was not detected in the other fractions (Fig. 1) containing 1 unit of DNA gyrase inhibited supercoiling of relaxed pBR322, whereas addition of fraction 25 did not (inset).

To investigate whether this inhibition was caused by nuclease activity, the 18-kDa protein (3 μg/ml) was incubated in the presence of relaxed or supercoiled pBR322 DNAs for 2 h at 37 °C and subsequently analyzed by agarose gel electrophoresis. Neither of the plasmid DNAs was degraded by the 18-kDa protein (data not shown). Furthermore, we examined the protease activity of the purified 18-kDa protein using subunits A and B of DNA gyrase as substrates. Their size and the amount of DNA gyrase subunits A and B were not influenced by the addition of the 18-kDa protein when examined by SDS-PAGE (data not shown). The above results suggested that the 18-kDa protein had neither nuclease nor protease activity.

Taken together, the results strongly suggested that the 18-kDa protein was an inhibitor of the supercoiling activity of DNA gyrase. We tentatively termed it DNA gyrase inhibitory protein (GyrI).

Analysis of NH₂-terminal Amino Acid Sequence of GyrI Protein—The NH₂-terminal amino acid sequence of the GyrI protein was determined to be MNYEIKQEEKTVAGF. Homology searching of protein data bases revealed that a putative gene product (157 amino acid residues, 18,095 Da) encoded by yeeB of E. coli had the same NH₂-terminal sequence (EMBL U00009). The function of this gene product has not been characterized to date. yeeB was mapped at 44 min of the E. coli genetic map, near the sbcB gene. Another synonymous gene (sbmC) encoding the same NH₂-terminal amino acids as GyrI, had been characterized as a gene that confers resistance to microcin B17 on E. coli when overexpressed (34). Microcin B17, a peptide antibiotic of 43 amino acids, induced breakage of double-stranded DNA in a DNA gyrase-dependent manner.

Exogenous Expression and Purification of 18-kDa Protein—We assumed that the gene encoding GyrI might be identical to yeeB and sbmC, and we amplified the gene based on the DNA sequence of the sbcB region by polymerase chain reaction. The amplified DNA was inserted into the pLEX vector, resulting in construction of pCA20.

E. coli GI724 was transformed with pCA20 and grown in RM medium containing 1% (w/v) glycerol and 2% (w/v) casamino acids. Induction of the expression of inserted gene was achieved by the addition of 100 μg of tryptophan/ml to the growth medium, and cultivation was continued for 5 h. SDS-PAGE analysis identified a Coomassie Blue-stained band of 18 kDa in cell lysate from tryptophan-induced E. coli containing pCA20. In contrast, the protein band was absent from the lysate of uninduced cells. The 18-kDa protein was purified by the procedures of ammonium sulfate fractionation, Sepharose Q column chromatography, and TSKgel filtration (Fig. 2A). A yield of 0.55 mg/liter of culture of the purified 18-kDa protein was obtained.

Inhibition of DNA Supercoiling Activity by 18-kDa Protein—The inhibitory effects on DNA gyrase by the purified 18-kDa protein are shown in Fig. 2B. The 18-kDa protein-free control (Fig. 2B, lane 1) contained relaxed pBR322 DNA and 1 unit of the DNA gyrase holoenzyme. The 18-kDa protein dose-dependently inhibited DNA supercoiling at doses of 2–8 μg/ml with complete inhibition at 8 μg/ml (Fig. 2B). The purified 18-kDa protein from the yeeB-overexpressing transformant inhibited DNA gyrase with a potency similar to that of the protein purified from E. coli KL16. Thus, we concluded that the gene encoding GyrI was identical to yeeB and sbmC. We tentatively named this gene gyrI to correlate the names of the gene and the gene product.

Effect of Overexpression of gyrI on Growth of E. coli—To investigate whether the expression of gyrI has an effect on cell growth, we used the expression plasmid pCA20, which contains the former. Colony-forming units (CFU) of the transformants carrying the plasmid (pCA20) were normal when the cells were grown in the absence of induction of gyrI expression (absence of tryptophan) (Fig. 3). Tryptophan was added to the culture at
when cultured in the absence of tryptophan (Fig. 4A), whereas using ethidium bromide staining of the nucleoids (Fig. 4). The morphological phenotypes of the transformants were examined influenced by the addition of tryptophan (data not shown). The CFU of transformants carrying the vector pLEX were not reduced from 10^7 to 10^6 CFU/ml 5 h after the addition of tryptophan, containing ampicillin (100 μg/ml). The positions of intracellularly negatively supercoiled and relaxed pBR322 DNA without 18-kDa protein and DNA gyrase are presented as shown.

Panel A, inhibition of supercoiling activity of DNA gyrase by the purified 18-kDa protein. Lane 1, control without 18-kDa protein; lanes 2, 3, and 4 are 18-kDa protein at 2, 4, and 8 μg/ml, respectively. Lane 5, control relaxed and lane 6, supercoiled pBR322, DNA are indicated by arrows. The pure 18-kDa protein was obtained from the E. coli strain overproducing GyrI. For details, see "Experimental Procedures."

Panel B, inhibition of supercoiling activity of DNA gyrase by the purified 18-kDa protein. Lane 1, control without 18-kDa protein; lanes 2, 3, and 4 are 18-kDa protein at 2, 4, and 8 μg/ml, respectively. Lane 5, control relaxed and lane 6, supercoiled pBR322, DNA are indicated by arrows. The pure 18-kDa protein was obtained from the E. coli strain overproducing GyrI. For details, see "Experimental Procedures."

FIG. 2. Purification of 18-kDa protein and inhibitory activity of purified 18-kDa protein against DNA gyrase. Panel A, purification of 18-kDa protein. Protein samples from each step of the purification were subjected to SDS-PAGE (12%). Lane 1, crude extract; lane 2, a dialyzed sample after ammonium sulfate precipitation; lane 3, pooled fractions after Q-Sepharose Fast Flow chromatography; lane 4, pooled fractions after TSKgel TOYOPEARL HW-55 chromatography; M, molecular mass markers. Lane 5, control relaxed and lane 6, supercoiled pBR322 DNA without 18-kDa protein and DNA gyrase are presented as shown.

When E. coli GI724 transformed with plasmid pCA20 that contained gyrI gene, increased from 2 × 10^7 to 1.4 × 10^9 CFU/ml. In the presence of tryptophan, the CFU did not change or tended to decrease. The bacterial cells carrying pCA19, which contained antisense gyrI gene, increased from 2 × 10^7 to 1.4 × 10^9 CFU/ml. The presence of tryptophan, the CFU did not change or tended to decrease. The bacterial cells carrying pCA19 exhibited filamentous growth in the presence of tryptophan as in the case of overexpression of gyrI (data not shown).

Effect of Expression of Antisense gyrI Gene on Growth of E. coli—We next assessed the physiological importance of GyrI by the means of expression of antisense gyrI gene (Fig. 5). To confirm that synthesis of GyrI was actually inhibited by the antisense gyrI we carried out Western blotting analysis with anti-GyrI antibody. Antibody-reactive proteins were not detected in the cells when expression of antisense gyrI was induced (data not shown). In the absence of tryptophan, the CFU of transformants carrying the plasmid pCA19, which contained antisense gyrI gene, increased from 2 × 10^7 to 1.4 × 10^9 CFU/ml. In the presence of tryptophan, the CFU did not change or tended to decrease. The bacterial cells carrying pCA19 exhibited filamentous growth in the presence of tryptophan as in the case of overexpression of gyrI (data not shown).

Expression Profile of gyrI Gene during Cell Growth—To investigate the expression of gyrI during cell growth, we constructed a plasmid, pCA15, which carried a gyrI-lacZ fused gene. The fused gene consisted of a putative promoter and NHL-terminal region of gyrI (~251 to 33-bp region), which contained a palindromic structure of s-factor-dependent terminator for yeeC 5′-adjacent to gyrI, a typical consensus sequence of −35 and −10 region for gyrI, and the structural gene of lacZ fused in a proper reading frame. E. coli JM109 transformed by
pCA15 was grown in LB medium at 37 °C, and aliquots of the culture were taken periodically during the growth transition from the exponential growth phase to stationary phase. The level of expression of GyrI, started to increase at the late exponential phase and reached the maximum level in the stationary phase (Fig. 6A).

Next, we examined synthesis of GyrI in E. coli KL16 during cell growth by a quantitative dot-blot assay using polyclonal antibodies against GyrI (Fig. 6B). The relative amounts of GyrI protein were determined by scanning the blots with a densitometer. The content of the protein increased about 2-fold/cell mass when the comparison was made between the cells of 0.75 and 1.3 A$_{600}$.

DISCUSSION

We purified from E. coli KL16 the 18-kDa protein that inhibited the supercoiling activity of DNA gyrase and coined it DNA gyrase inhibitory protein (GyrI). The NH$_2$-terminal amino acid sequence and molecular mass of GyrI inferred that the gene encoding GyrI might be identical to the previously reported genes of yeeB (EMBL accession no. U00009) and sbmC (34). The yeeB gene had been identified as an open reading frame in the sbcB region, although its function had not been described. The sbmC gene had been reported to decrease the sensitivity to microcin B17 when overexpressed. Microcin B17, a peptide antibiotic of 43 amino acids, is generated by cleavage of a precursor of 69 amino acids encoded by sbmC and appears to trap an abortive cleavable DNA-DNA gyrase complex (30), a mode of action similar to that of quinolones. The yeeB and sbmC gene was located at 44 min on the E. coli chromosome map. However, there have been no reports on the inhibitory activity against DNA gyrase of the gene products of yeeB and sbmC.

To investigate the identity of gene encoding GyrI with yeeB and sbmC, we cloned the coding region based on the reported sequence and purified the 18-kDa gene product from the transformant overexpressing it. In vitro assay of DNA gyrase supercoiling activity indicated that the purified 18-kDa protein indeed inhibited the activity. Furthermore, we confirmed that GyrI protein is not intercalated into DNA and does not inhibit the activity of other DNA-processing enzymes (e.g. DNA polymerase) (data not shown). We tentatively named the gene coding for the 18-kDa protein as gyrI to indicate clearly the biological importance of the function of the gene product.

It was reported that factor LetD regulates the activity of DNA gyrase (13). LetD encoded by the F factor functions to kill the host E. coli (37–39). The killing effect of LetD is suppressed by a mutation in gyrA or by overexpression of gyrA, suggesting that one target of LetD protein in cells is DNA gyrase (13). This has been attributed to the following mechanism. Expression of LetD protein leads to synthesis of $\sigma$32, which induces DnaK and GroEL proteins, thus inhibiting DNA gyrase activity (15, 40). In contrast to LetD, GyrI is the first identified regulatory factor for DNA gyrase which directly inhibits the activity in vitro and is encoded on the chromosome of E. coli.

To assess the in vivo importance of the function of GyrI, we
examined the morphological phenotype of cells with perturbed expression of gyrI using overexpression of gyrI itself or antisense gyrI. Overexpression of gyrI and expression of antisense gyrI suppressed proliferation of the host cells and decreased the number of the viable cells. Microscopic examination revealed that some population of the cells overexpressing sense or antisense gyrI grew filamentously and had nucleoids with abnormal morphology as described above. The abnormal shapes of cells and nucleoids were similar to those observed in bacterial cells treated with quinolones (41), suggesting that the abnormality might be caused by perturbation of DNA gyrase activity in the cells expressing sense or antisense gyrI. Thus, it is conceivable that GyrI is involved in regulation of DNA gyrase in vivo.

The promoter activity of gyrA, the gene coding for the subunit A of DNA gyrase, increases in the mid-exponential phase to peak in the late exponential growth phase and thereafter decreases to the level of that in the mid-exponential phase (14). In contrast, transcription of gyrI is expressed mainly from the late growth phase to the stationary phase, as assessed by using the reporter system. By dot-blot assay with the anti-GyrI antibody, it was shown that GyrI was synthesized in a pattern similar to that of transcription of gyrI during cell growth. There was found at the 5' region (~36 to ~31) of gyrI a consensus sequence (TATACT) for recognition by transcription factor ω38, which specifically functions for gene expression in the late growth phase (42). To confirm involvement of ω38 in transcription of gyrI, further studies will be needed. The concerted regulation of expression of the genes (gyrA and gyrB) encoding DNA gyrase subunits and the gene encoding the regulatory factor (gyrI) of DNA gyrase might be critical for DNA replication and cell proliferation.

This study demonstrated that disturbance (reduction or amplification) of GyrI levels resulted in suppression of bacterial cell proliferation. gyrI/GyrI might be novel and promising targets for development of new antibacterial agents.

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