Modulation of DNA Supercoiling Activity of Escherichia coli DNA Gyrase by F Plasmid Proteins

ANTAGONISTIC ACTIONS OF LetA (CcdA) AND LetD (CcdB) PROTEINS*

(Received for publication, October 11, 1991)

Satoko Maki, Souichi Takiguchi, Takeyoshi Miki, and Tadao Horiuchi

From the Department of Microbiology, Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi, Fukuoka 812, Japan

The letA (ccdA) and letD (ccdB) genes of F plasmid contribute to stable maintenance of the plasmid in Escherichia coli cells; a product of the latter has a lethal effect on the host cell and that of the former neutralizes functions of the letD. In cells that overproduce the LetD (CcdB) protein, the plasmid DNA is extensively relaxed. Correspondingly, DNA supercoiling activity in a cell-free extract of the overproducing strain decreases to a level of less than 1% of that seen in normal cells. However, the extract does not inhibit DNA gyrase reconstituted from purified subunits, thereby indicating that the intrinsic DNA gyrase is inactivated in the overproducing strain. Upon addition of purified LetA (CcdA) protein to the extract of LetD overproducing cells, the DNA supercoiling activity was fully restored. Using this rejuvenation as an assay, we purified the “inactivated gyrase” and obtained evidence that the LetD protein formed an isolable complex with the A subunit of DNA gyrase. Thus, the LetD and the LetA proteins constitute an opposing pair in modulating the DNA supercoiling activity of gyrase, probably by direct interaction.

The ccd operon of F plasmid, located in the primary replicon region (repFIA), consists of letA (ccdA), letD (ccdB), and resD genes and contributes to stable maintenance of the plasmid in Escherichia coli. The letD gene product (11.7 kDa) has a lethal effect on the host cell and the letA gene product (8.3 kDa) functions in neutralizing the letD function (1–4). Cells in which the letD but not the letA gene is expressed form fila-ments with large masses of unseparated nucleoids irregularly distributed in the cells. Comitont formation of small anucleate cells was also observed (4, 5). The same phenotype can be elicited by blocking replication of the F plasmid carrying the intact letA and letD genes (3). Jaffé and colleagues (5) found that the letD function is to inhibit division and cause death specifically of plasmid-free segregants arising from failure of replication or partition, thereby assuring survival of only F+ cells retaining the letA function (nonviable segregant model) (5, 6). However, the molecular basis for the inhibition of host cell growth by LetD protein and the suppression by LetA protein is poorly understood.

The LetD protein induces SOS functions although the killing activity of the protein is exhibited in both recA+ and recA− bacteria (4, 7–9). From studies of this LetD-mediated SOS induction, Bailone et al. (10) speculated that the SOS signal resides on the host chromosome rather than on the F DNA and further postulated that the LetD protein might inhibit chromosome decatenation by DNA gyrase, leading to generation of single-stranded DNA by the action of RecBCD helicase (10, 11). Phenotypic similarities between some gyrase mutants and the letD-expressing cells support this notion. E. coli par mutants (12) show basically the same nucleoid morphology and filamentous cell growth as noted above, under restrictive conditions. The Par phenotype of parA and parD mutants have been ascribed to gyrb(,,) and gyrA(,,) mutations, respectively (13, 14). The mutants are considered to have lost control over the spatial location of septa, probably as a result of a primary defect at separation of the intertwined chromosomes after DNA replication (15). It may be that the LetD protein hampers cellular segregative machinery in which the par genes are involved.

It attempts to obtain clues for target protein(s) of the LetD protein, mutants that are tolerant to the inhibitory effect of LetD protein (tld mutants, tolerance to letD product growth inhibition) have been isolated (16). We found one such mutant (tldC15) in gyrA, a structural gene for the A subunit of DNA gyrase (17). The mutant was able to form colonies under LetA− LetD+ conditions at 28 °C and the mutation itself rendered the cell temperature sensitive. The mutant GyrA protein was transdominant over wild type GyrA protein for LetD tolerance. In addition, increased dosage of the wild type gyrA gene overcame the growth inhibition of the letD gene product. These observations suggested that action of the LetD protein is on DNA gyrase.

We report here that the target of LetD protein is DNA gyrase, particularly the A subunit. In the LetD overproducing cells, DNA gyrase (A,B), as well as a free form of the A subunit, which was shown to be present in 20-fold excess over the A2B2 tetramer, existed in an inactivated form. This inactivation is likely due to binding of the LetD protein to the A subunit. The LetA protein is able to rejuvenate the inactivated gyrase or the A subunit.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The E. coli strain KP3998 (lacF) has been described elsewhere (18). Plasmid pMJR1560, a source of a lacF fragment, was kindly provided by M. Imai, Kyoto University. pKP1498 (18) contains a fragment carrying letA gene inserted into BamHI and HincII sites of a tac promoter-mediated expression vector pKP1500 (18). pKP1052, a pBR322 (19) derivative carrying the EcoRI

* This work was supported in part by grants-in-aid for Scientific Research on Priority Areas 03221102, 03262111 (to T. M.) and grants-in-aid for Scientific Research 01790558 (to S. M.) from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
fragment G (Spc') of NRI (20), was constructed in our laboratory, pKP1444, a pUC9 (21) derivative constructed in our laboratory,1 contains a fragment carrying the Spc' gene derived from pKP1052 and a BamHI-PstI fragment carrying letA-letD genes of F13-1 (42.84-43.6F) (4). A new EcoRI site was introduced just upstream of the initial codon of the letD gene, using a nucleotide-directed site-specific mutagenesis. pKP1110 is a pUC9 derivative carrying the EcoRI fragment G of NRI.

Reagents and Proteins—Sources were: IPTG,2 ATP, creatine phosphokinase (rabbit muscle, type I), phosphocreatine (di-Tris salt), and E. coli tRNA, Sigma; spermine, Wako; bovine serum albumin (Fraction V), Boehringer Mannheim; protein standards for SDS-PAGE (high and low molecular weight range packages), and topoisomerase I (calf thymus), Bethesda Research Laboratories; proteins K, E. Merck; agarose, T4 DNA ligase, and restriction enzymes, EcoRI, HindIII, PstI, and BamHI, Takara; gel filtration calibration kit, Pharmacia LKB Biotechnology Inc.; Vectastain Elite ABC kit (avidin biotinylated horseradish peroxide H2O2, biotinylated anti-rabbit IgG) and substrate kit (3,3'-diaminobenzidine, 4-chloro-l-naphthol), Vector Laboratories; E. coli GyrA, GyrB, and topoisomerase I proteins, gifts from S. Sekimu, University of Tokyo; rabbit antiserum against GyrA protein, gift from J. Kato, University of Tokyo; rabbit antiserum against E. coli GyrA and GyrB, and topoisomerase I proteins was purified, in our laboratory;2 LetA-letD complex, purified in our laboratory. The LetA, LetD, and LetA-letD complex purified proteins were purified to apparent homogeneity from their overproducing strains by following the proteins by SDS-PAGE (detailed procedures will be described elsewhere). N-terminal amino acid sequences of the purified proteins were determined and they agreed with those deduced from the DNA sequences of the corresponding genes.

Construction of letD Overproducing Plasmid—Three steps were taken to construct a LetD overproducing plasmid: (i) construction of a LetD-LetA overproducing plasmid; (ii) introduction of the lacP gene into the plasmid; and (iii) deletion of the letA gene. First, a HindIII fragment of pKP1052 was inserted into an EcoRI site of pJM1505 in order to provide a selective marker for E. coli strains and the control strain. The resulting plasmid, pKP1874, was used to overproduce the LetD protein.

A control plasmid lacking the letD gene was constructed by inserting a HindIII site in pKP1505 located just downstream of the letA gene, and pKP1876 was obtained. Third, a PstI fragment carrying the letA gene was deleted from the plasmid (pKP1876) that has two PstI sites, one in the multicloning site at the junction of the letD and letA genes and the other in the downstream of the letA gene. The final construct, pKP1878, was not successfully introduced into E. coli strain KP3998 (lacP). Instead, the plasmid was introduced into KP3998 harboring F' lac (KP6261) and the strain (KP6261/pKP1878) was used to overproduce the LetD protein.

When an ATP-regenerating system consisting of creatine kinase and creatine phosphate was not included in the assay, activity that of KP5621/pKP1879. Cell lysates were prepared using lysozyme and heat treatment (37 °C) in the presence of spermidine as described (32).

Topological Analysis of Isolated Plasmid—After KP6261/pKP1878 and KP6261/pKP1879 were grown under the conditions specified, they were chilled rapidly by mixing with an equal volume of frozen medium, and plasmid DNAs (pKP1878 and pKP1879) were isolated by the alkaline lysis method with proteinase K treatment (23). The DNAs were electrophoresed on a 0.8% agarose gel (14 × 12.5 × 0.69 cm) in Tris borate/EDTA buffer (23) at 1.6 V/cm for 14 h. The gel was stained with 1 μg/ml ethidium bromide and photographed under ultraviolet light on Polaroid type 55 film.

In Vitro DNA Supercoiling Assay—The assay measures conversion of the relaxed closed-circular form of pKP1110 DNA to the supercoiled form, as demonstrated by agarose gel electrophoresis. The substrate DNA was prepared by treating the supercoiled form of the DNA with calf thymus topoisomerase I according to the manufacturer's instruction. The activities of gyrase (A,B)2, GyrA, and GyrB in cell-free extracts were selectively measured by addition of none, excess purified GyrB, and excess purified GyrA, respectively. The purified GyrA and GyrB proteins were free of contaminating GyrB and GyrA activities, respectively. The standard reaction mixture (19.5 μl) contained: 35 mM Tris-HCl (pH 7.5), 0.5% (w/v) glycerol, 0.14 mM EDTA, 4 mM MgCl2, 24 mM KCl, 1.8 mM spermidine- HCl, 1.4 mM ATP, 0.1 mg/ml creatine kinase, 8 mM creatine phosphate, 0.9 μg/ml E. coli tRNA, 0.36 mg/ml bovine serum albumin, 5 mM dithiothreitol, 0.24 μg (43 fmol of DNA molecule) of the relaxed closed-circular DNA, and enzyme fractions to be assayed. The components above, except for the enzyme fractions, were combined in a total volume of 14.5 μl and preincubated for 5 min. The enzyme mixture (6 μl) containing cell lysates, GyrA, or GyrB, was preincubated at 25 °C for 5 min. Reaction was initiated by addition of the premix into the enzyme mixture, left to proceed at 25 °C for 60 min, and terminated by adding EDTA (10 mM) and SDS (0.5%). The product DNA was electrophoresed and the gel was photographed as above. One unit of DNA supercoiling activity is defined as converting 50% of the DNA in the standard assay to supercoiled species which migrate at the fully supercoiled position, as measured by a densitometric scan (2202 Ultrascan laser densitometer, LKB) of negatives of the photographs (24). When the activities were too low to be able to measure directly, the supercoiling activity was determined by comparing the patterns of DNA on agarose gels with those produced by activity of known units. Enzymes were diluted when necessary into 50 mM Tris-HCl (pH 7.5), 5.0% (w/v) glycerol, 1 mM EDTA, 50 mM KCl, 3.6 mg/ml bovine serum albumin, and 5 mM dithiothreitol.

When an ATP-regenerating system consisting of creatine kinase and creatine phosphate was not included in the assay, activity that inhibited DNA gyrase was detected in E. coli cell extracts. We purified this activity to apparent homogeneity and it proved to be glycerol kinase (EC 2.7.1.30) encoded by the glpK gene located at 88 min on the E. coli genetic map. The enzyme catalyzes phosphorylation of glycerol which in turn generates ADP from ATP. We found this ADP production to be inhibitory to the supercoiling activity of DNA gyrase.

Cell Growth and Preparation of Cell Extracts—Since the growth of E. coli cells was affected by a very low level of production of the LetD protein even in the presence of F' lac, plasmid pKP1878 was easily lost from the cells during growth, resulting in a poor production of LetD protein. To overcome this problem, E. coli KP5621/pKP1878 was first grown on nutrient agar plates containing ampicillin (100 μg/ml) and spectinomycin (50 μg/ml) at 28 °C. Cells forming colonies were scraped off with a sterile spatula and inoculated into 450 ml of PYGMN medium (1% polypeptide, 0.5% yeast extract, 0.5% glycerol, 0.5% NaCl, 0.01% MgSO4) in a 2-liter flask so as to adjust an initial A695 of 0.6, at which point the temperature was shifted to 40 °C. After 1 h, IPTG was added to the culture to a final concentration of 2 mM, and the cells were left to grow at 40 °C for another 0.5, 1.0, and 1.5 h, depending on the experiment. The cells were harvested by centrifugation, suspended to A695 of 400 in 50 mM Tris-HCl (pH 7.5) containing 10% sucrose, and frozen immediately in liquid nitrogen. The frozen cells were lyophilized for the same period.
grown under full induction conditions, was treated with ammonium sulfate (0.35 mg/ml of cell extract) and the precipitate was collected by centrifugation (31,000 x g, 0 °C, 60 min). The suspension of the pellet (4 ml, 64 mg of protein) in buffer A (50 mM Tris-HCl (pH 7.5), 15% (v/v) glycerol, 0.1 mM EDTA, 5 mM dithiothreitol) was dialyzed against the same buffer and loaded onto a 3-5 ml DEAE-trisacryl (type M, Pharmacia) column equilibrated with buffer A + 50 mM KCl. The column was washed with 5-column volumes of the equilibration buffer and the activity was step eluted by 5-column volumes of buffer A containing cells were killed immediately after the addition of IPTG to the medium, which induced transcription from the tac promoter. Upon induction, a polypeptide of 11 kDa was observed in the gelatinase assay (Fig. 2A). The expression of the letD gene depends on the tac promoter and the lacZ Shine-Dalgarno sequence. To ensure a tight repression of the transcription of the letD gene, the lacZ gene was cloned after the temperature shift were analyzed by SDS-PAGE with Coomassie Blue staining. B, optical densities (Abs) were followed before and after the temperature shift (C). Viable cells containing plasmid pKP1878 were counted by plating samples on nutrient agar plates containing ampicillin (25 μg/ml) and spectinomycin (15 μg/ml) at 28 °C (∗).

**RESULTS**

**Amplification of the letD Gene Product**—The letD gene of F plasmid was cloned on a expression vector derived from plasmid pUC9 (18). In the resulting plasmid (pKP1878), expression of the letD gene depends on the tac promoter and the lacZ Shine-Dalgarno sequence. To ensure a tight repression of the transcription of the letD gene, the lacZ gene was cloned after the temperature shift were analyzed by SDS-PAGE with Coomassie Brilliant Blue. These experiments were essentially as described in (27). 15% SDS-polyacrylamide gels were used for analyses of LetD and LetA proteins and 12.5% gel for gyrA protein. Protein concentration was determined by the method of Bradford (28) with bovine serum albumin as a standard. Enzymes and cell lysates stored in small aliquots at −80 °C showed no changes in activity.

**Immunological Detection of GyrA and LetD Proteins**—The immunoblot method of Burnette (26) was carried out, but with modifications. Proteins resolved by SDS-PAGE were transferred electrophoretically onto membranes (Immobilon PVDF (Millipore) for detection of GyrA protein and Zeta-Probe (Bio-Rad) for detection of LetD protein by a semi-dry blotter (Trans-Blot SD, Bio-Rad) following the manufacturer’s instructions. The blots were blocked in a solution of 5% skim milk (Difco) in Tris-saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and then incubated in the primary antibody diluted in an antibody buffer (1% skim milk in Tris-saline) for at least 2 h followed by three washes in Tris-saline containing 0.05% Tween 20. The anti-GyrA and the anti-LetA sera were diluted 50- and 50-fold, respectively. The blots were incubated next with a biotinylated anti-rabbit IgG (Vector Laboratories), at a dilution of 1 in 2000, in antibody buffer and then with an avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) in antibody buffer with washes in Tris-saline/Tween 20 between the incubations. After a thorough washing twice with Tris-saline/Tween 20 and once with Tris-saline, the blots were subjected to visualization reactions. 4-Chloro-1-naphthol (for detection of GyrA) and 3,3′-diaminobenzidine (for detection of LetD) were used as substrates for horseradish peroxidase, according to the manufacturer’s instructions (Vector Laboratories). All operations were carried out at ambient temperature.

**Other Methods**—Isolation of plasmid DNA, plasmid construction, and transformation were as described (23). SDS-PAGE and gel staining with Coomassie Brilliant Blue were essentially as described (27).

**DNA Gyrase**—We first measured the superhelical density of DNA gyrase, we first measured the superhelical density of plasmid DNA in the LetD overproducing cells. The IRtD gene product was achieved by two methods—Isolation of plasmid DNA, plasmid construction, and transformation were as described (23).

**Amplification of the letD Gene Product**—The letD gene of F plasmid was cloned on an expression vector derived from plasmid pUC9 (18). In the resulting plasmid (pKP1878), expression of the letD gene depends on the tac promoter and the lacZ Shine-Dalgarno sequence. To ensure a tight repression of the transcription of the letD gene, the lacZ gene was cloned after the temperature shift were analyzed by SDS-PAGE with Coomassie Blue staining. B, optical densities (Abs) were followed before and after the temperature shift (C). Viable cells containing plasmid pKP1878 were counted by plating samples on nutrient agar plates containing ampicillin (25 μg/ml) and spectinomycin (15 μg/ml) at 28 °C (∗).

**RESULTS**

**Amplification of the letD Gene Product**—The letD gene of F plasmid was cloned on an expression vector derived from plasmid pUC9 (18). In the resulting plasmid (pKP1878), expression of the letD gene depends on the tac promoter and the lacZ Shine-Dalgarno sequence. To ensure a tight repression of the transcription of the letD gene, the lacZ gene was cloned after the temperature shift were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining. B, optical densities (Abs) were followed before and after the temperature shift (C). Viable cells containing plasmid pKP1878 were counted by plating samples on nutrient agar plates containing ampicillin (25 μg/ml) and spectinomycin (15 μg/ml) at 28 °C (∗).
the same host strain carrying the plasmid grown under the same condition (Fig. 2B). Upon the addition of IPTG, the plasmid DNA in the LetD overproducing cells became extensively relaxed. It should be noted that the relaxation was readily recognized in an agarose gel, without chloroquine. In contrast, the control plasmid remained supercoiled throughout the time course. Therefore, relaxation of the plasmid was attributed to large amplification of the LetD protein. Copy number of the control plasmid increased, as expected after heat treatment, but there was no such increase with the overproducing plasmid. This may have occurred because DNA replication, in which an action of DNA gyrase is required, was halted after adding IPTG. However, since the level of amplification of the LetD protein exceeded that explained solely by derepression of the tac promoter, it is equally probable that an unusually tangled plasmid DNA due to loss of decatenation activity of DNA gyrase could not be isolated using alkaline lysis.

Complete Disappearance of DNA Gyrase Activity in the LetD Overproducing Cells—Cell-free extracts were prepared from the LetD overproducing strain (KP5621/pKP1878) and its control strain (KP5621/pKP1879), and DNA supercoiling activity in the extracts was assayed. The extract from the control strain catalyzed conversion of relaxed closed-circular DNA to faster migrating species and finally to a form whose electrophoretic mobility indistinguishable from that of naturally supercoiled DNA. On the other hand, no topological change was observed when comparable amounts of the extract from the overproducing strain were titrated in the reaction (Fig. 3A). As shown in Fig. 3B, DNA supercoiling activity in the extract of LetD overproducing strain was retained before the induction and was dramatically lowered to a nondetectable level in a manner dependent on the expression of the letD gene. Thus, it is evident that the loss of supercoiling activity was caused by the overproduction of LetD protein.

One possible explanation for the disappearance of DNA supercoiling activity is enhancement of the DNA relaxing activity such as that of topoisomerase I of E. coli. However, no significant DNA relaxing activity was observed either in the extract of the overproducing strain or in that of the control strain, when assayed under the same condition as for the DNA supercoiling reaction (data not shown). Alternatively, the disappearance of supercoiling activity could be due to activity inhibiting DNA gyrase. When DNA gyrase reconstituted from its purified subunits was incubated with the extract of LetD overproducing strain in the DNA supercoiling assay, no such inhibition was detected (data not shown, see “Experimental Procedures”). These observations strongly suggest that the DNA gyrase activity had disappeared from cells upon induction of the LetD protein.

DNA gyrase is a tetramer of two each of A (GyRA) and B (GyRB) subunits (29). To determine which of the subunits was affected, GyRA and GyRB activities in the cell extracts were assayed separately (Table I). In the extract of the control strain, the total GyRA activity (assayed in the presence of purified GyRA in excess) was equal to that of the A₂B₂ tetramer (assayed in neither presence of GyRA nor GyRB). Therefore, almost all the active GyRB protein was present in the A₂B₂ complex. The total GyRA activity (assayed in the presence of excess GyRB) was 20-fold higher than that of the A₂B₂ tetramer, thereby indicating that 20 times more GyRA protein existed in a free form than that in the A₂B₂ complex. Compared with these activities, the activities of both GyRA (including the free form) and GyRB almost completely disappeared in the extract of LetD overproducing cells.

Rejuvenation of Inactivated DNA Gyrase by LetA Protein—If the disappearance of the DNA gyrase activity in the LetD overproducing cells was due to inactivation of the protein but not to its degradation or repression of the gyr genes, the inactivated gyrase may be rejuvenated. Since LetA protein neutralizes the lethal effect of LetD protein in vivo (1, 4), we tested whether the DNA supercoiling activity in the extract of LetD overproducing cells could be restored by adding purified LetA protein to the extract. As shown in Fig. 4, we observed the LetA-dependent conversion of relaxed closed-circular DNA to forms with increased mobility on an agarose gel. The LetA protein itself exhibited neither DNA supercoiling activity (Fig. 4) nor stimulation of supercoiling by the purified DNA gyrase (data not shown). A maximal restoration of the supercoiling activity was obtained at roughly a 1:1 ratio of LetA to LetD, assuming that 20% of the total protein in the extract is the LetD protein. The restoration capacity of LetA protein was further confirmed by carrying out the assay with fractions of DEAE-5PW HPLC column chromatography.

![Fig. 3. Disappearance of DNA supercoiling activity upon overproduction of LetD protein. DNA supercoiling activity in cell extracts was assayed as described under “Experimental Procedures.” A, KP5621/pKP1878 and KP5621/pKP1879 were grown as described in the legend for Fig. 1. From cells harvested after 2.5 h after the temperature shift, extracts were prepared and the cell extracts were titrated in the DNA supercoiling assay. B, KP5621/pKP1878 grown as described were harvested at 0, 1, 1.5, 2, and 2.5 h after the temperature shift and extracts were prepared. 3 μg of each cell extract were assayed for their supercoiling activity.](image-url)

**Table I. DNA supercoiling activities in cell extracts of LetD overproducing and control strains**

| Activity | LetA protein | Gyrase (A₂B₂) | GyrB subunit | A subunit | GyrB (A₂B₂) | A subunit | Inactivation (%) | Restoration (%) |
|----------|--------------|---------------|--------------|-----------|-------------|-----------|-----------------|-----------------|
|          |              | Superoiling activity | Control | LetD overproducer |              | units × 10⁻³ | mg protein |              |                  |
| Gyrase (A₂B₂) | - | 1.0 | <0.01 | <1 | GyrB subunit | 1.2 | 0.01 | 1 |                  |
| A subunit | - | 22 | 0.2 | 1 | GyrB (A₂B₂) | + | 1.0 | 0.5 | 50 |
| B subunit | + | 1.2 | 0.5 | 42 | A subunit | + | 22 | 20 | 91 |

* a DNA supercoiling activity in cell extract of the LetD overproducing strain relative to that in extract of the control strain.

* b Restored supercoiling activity in cell extract of the LetD overproducing strain relative to DNA supercoiling activity in extract of the control strain.
of DNA gyrase (data not shown). These results strongly suggest that an inactivated form of DNA gyrase was present in the LetD overproducing cells and that the LetA protein had a capacity to rejuvenate it. Further titration of the LetA protein in the rejuvenation assay showed an inhibitory effect (Fig. 4). Whether this inhibition is on the rejuvenation process or on the action of DNA supercoiling remains to be examined. The LetA protein itself did not inhibit supercoiling activity of the purified DNA gyrase even when an excess amount of the protein was added (data not shown).

The extent of the restoration by LetA protein is summarized in Table I. When the LetA protein was added to the extract from the control cells, there was no stimulation of the DNA supercoiling activity. In contrast, the addition of the LetA protein to the extract of LetD overproducing cells restored the DNA gyrase activity to a level comparable with that seen in the control extract; the activity of A,B,C complex (assayed in the presence of none or GyrA in excess) was restored to 40–50% of that in the control extract. Moreover, the activity of free GyrA protein (assayed in the presence of GyrB in excess) was restored to a level as high as 90% of that in the control extract. It seems, therefore, that the primary target of the LetD protein is the A subunit of DNA gyrase. When the A subunit in the A,B,C complex is inactivated, the B subunit probably remains in the complex so that its activity is not detectable even in the presence of active GyrA protein. The LetA protein has the capacity to rejuvenate the inactivated form of the GyrA protein whether it is in a free form or in the A,B,C complex.

Association of LetD Protein with the Inactivated A Subunit of DNA Gyrase—The restoration of supercoiling activity by the LetA protein made way for an assay to purify the inactivated gyrase in the LetD overproducing cells. DNA supercoiling activity that appeared in the presence of both LetA and GyrB proteins (LetA-GyrB-dependent DNA supercoiling activity, see Fig. 6) was followed since it was abundant and stable (Table I). The extract of LetD overproducing cells was fractionated first by ammonium sulfate precipitation. The LetA-GyrB-dependent supercoiling activity was recovered in the precipitate along with a large amount of the LetD protein. A significant portion of the supercoiling activity that appeared in the presence of only LetA protein (LetA-dependent DNA supercoiling activity, see Fig. 6) remained in the soluble fraction, however, the yield was low. The precipitate fraction was then subjected to a DEAE-trisacryl column chromatography. Almost all the LetD protein was recovered in a flow-through fraction of the chromatography, whereas the LetA-

![FIG. 4. Restoration of DNA supercoiling activity by LetA protein. The DNA supercoiling assay was carried out with a fixed amount (6.3 μg) of the cell extract of KP5621/pKP1878 used in Fig. 3A, and purified LetA protein was titrated in the assay. After the reactions were terminated, product DNAs were extracted with phenol-CHCl₃ and analyzed by agarose gel electrophoresis.](image)

![FIG. 5. Agreement of LetA protein peak with the peak of restored supercoiling activity in DEAE column chromatography. Peak fractions of LetA protein in the DEAE-5PW (TSK) HPLC column chromatography, a final step in purification of the LetA protein (detailed procedures will be described elsewhere), were assayed for their capacity to restore DNA supercoiling activity. A, protein concentration. B, samples (2 μl) of designated fractions shown in A were subjected to SDS-PAGE. Proteins were visualized by Coomassie Blue staining. C, the DNA supercoiling activities were measured with a fixed amount (6.3 μg) of the cell extract of KP5621/pKP1878 used in Fig. 3A and 0.5 μl each of designated fractions. Amounts of the LetA protein in the reactions were: 8 ng, fraction 50; 80 ng, fraction 51; 210 ng, fraction 52; 260 ng, fraction 53; 380 ng, fraction 54; 500 ng, fraction 55; 250 ng, fraction 56; 90 ng, fraction 57. Product DNAs were extracted with phenol-CHCl₃ and analyzed by agarose gel electrophoresis.](image)

![FIG. 6. LetA-dependent and LetA-GyrB-dependent supercoiling activities in the extract of LetD overproducing cells. The DNA supercoiling assay was carried out as described. Enzyme mixtures contained the cell extract of KP5621/pKP1878 used in Fig. 3A (3 μg for ++ and 0.1 μg for +), LetA (0.25 μg for ++ and 0.024 μg for +), GyrB (25 ng), and GyrA (0.6 ng) in combinations, as shown in the figure.](image)
GyrB-dependent supercoiling activity was step eluted with a buffer containing 300 mM KCl in a single peak with a recovery of 84%. This activity was then size fractionated by Superose 12 gel filtration. In the same experiment but under separate runs, the purified LetD and GyrA proteins as well as the standard protein markers were analyzed (Fig. 7). The LetA·GyrB-dependent supercoiling activity was recovered in a single peak with a 38% recovery. Stokes' radii of the LetA·GyrB-dependent supercoiling activity, GyrA protein, and the LetD protein were 68, 64, and 22 Å, respectively (Fig. 7A). It should be emphasized that the LetA·GyrB-dependent supercoiling activity was eluted at a position close to but corresponding to a somewhat larger molecule than that of the GyrA protein.

Among the many bands still present in peak fractions of the LetA·GyrB-dependent supercoiling activity visualized by silver staining of an SDS gel, two bands corresponding to the sizes of GyrA and LetD coincided with the LetA·GyrB-dependent supercoiling activity (data not shown). Location of GyrA and LetD proteins was therefore examined directly by immunoblotting. Fractions containing the LetA·GyrB-dependent supercoiling activity were subjected to SDS-PAGE, transferred to a membrane, and monitored with antibodies directed against GyrA and LetD proteins (Fig. 7, B and C). Two bands which migrated to the positions of GyrA and LetD proteins cross-reacted with the corresponding antibodies. These protein peaks coincided perfectly with the LetA·GyrB-dependent supercoiling activity. Stoichiometry of these proteins, calculated by comparing the immunoblots of known amounts of purified GyrA and LetD proteins serving as standards, was roughly 1 to 1. Taken together, these results strongly suggest that the A subunit of the DNA gyrase was inactivated in the LetD overproducing cells as a result of formation of a GyrA-LetD complex. Establishment of the components and stoichiometry of the complex awaits further purification.

**DISCUSSION**

We constructed a plasmid that allows a high level production of the LetD (CcdB) protein and we examined its effect on DNA supercoiling activity. Upon induction of the LetD protein, cells carrying the plasmid formed filaments and were killed. In these cells, DNA supercoiling activity was decreased to a nondetectable level, in a manner dependent on the production of LetD protein. Biochemical analyses of cell-free extracts of the LetD overproducing strain revealed that this was due to inactivation of intrinsic DNA gyrase. The extracts did not contain inhibitory capacity for an extrinsic DNA gyrase. A free form of A subunit of gyrase (GyrA), which was shown to exist in a 20-fold excess over the A<sub>B</sub><sub>T</sub> tetramer in normal cells, was also completely inactivated in the LetD overproducing cells. On the other hand, the LetA protein, genetically suggested to be a suppressor of the LetD function (1, 4), possessed the ability to rejuvenate the inactivated form of both DNA gyrase and GyrA protein. Addition of the LetA protein to the extract of LetD overproducing cells led to almost full rejuvenation of the inactivated proteins. Using this rejuvenation as an assay, the inactivated GyrA protein was partially purified. Gel filtration of the inactivated GyrA protein showed it to be somewhat larger than the active GyrA protein. Accordingly, a protein that cross-reacted with an antibody against the LetD protein was co-chromatographed with the inactivated GyrA protein, thereby implying association between the LetD protein and the GyrA protein. This complex formation might be the basis for the GyrA inactivation. Based on the present observations, we conclude that the A subunit of DNA gyrase is a primary target of the LetD protein and that disappearance of the DNA gyrase activity from the cell accounts for the killing effect of the LetD protein. Furthermore, the results indicate that the LetD and LetA proteins constitute an opposing pair in modulating the DNA supercoiling activity of the DNA gyrase; the LetD protein inactivates the GyrA protein probably by a direct interaction, whereas the LetA protein is capable of reversing the inactivation process.

Attempts to reconstitute *in vitro* the inactivation of gyrase using purified LetD protein or extract of the LetD overproducing cells have not succeeded, possibly because cofactors or conditions might be lacking. One possible explanation is that the inactivation process requires a chaperone function (31). The notion derives from observations that mutations in *groES* and *groEL* genes, whose products facilitate folding of newly synthesized polypeptides and their assembly into oligomeric structures (32), overcome the LetD-mediated killing (16, 17). In contrast to the inactivation of DNA gyrase, the reverse reaction was demonstrated *in vitro*; purified LetA protein possessed biochemical activity to rejuvenate the inactivated GyrA protein. Since the LetD and LetA proteins form a tight complex of 69 (64)<sub>4</sub> kDa (33), it is tempting to postulate that the LetA protein removes the LetD protein from a GyrA·LetD complex by forming a LetA-LetD complex. The assumption that the LetA-LetD complex is inert as an inactivator is consistent with the *in vivo* observation that cells expressing both *letA* and *letD* genes show no growth inhibition (1). However, it is unclear how the LetD protein in the LetA-LetD complex becomes free to be activated in cells that had lost the F plasmid. There is a report of instability of the LetA protein, and if this notion is valid, it would explain the activation of LetA·LetD complex (33). We observed no such instability with respect to the rejuvenation capacity of the
purified LetA protein during normal handling, although the question of stability of the protein was not directly addressed. Complete reconstitution of both inactivation and reactivation of DNA gyrase from purified proteins is necessary to clarify all components involved in the process, and their molecular mode of action.

While mechanisms by which the LetD protein inactivates gyrase remain to be determined, one differing from the inhibitory action of quinolone antibiotics (34), potent inhibitors of the A subunit of DNA gyrase, seems to be operative. Nalidixic acid and related compounds cause an immediate shut-off in DNA synthesis by trapping a covalently bound gyrase-DNA complex, which interferes with movement of the replication fork (35, 36). A biochemical view of the inhibition well explains a genetical observation that nalidixic acid-resistant gyrA mutation (Nal') is recessive against the wild type (Nal') allele (37). In contrast, under physiological circumstances in which the LetD protein functions to kill cells, the LetD protein shows no intriguing effect on DNA synthesis (5). Accordingly, a class of gyrA mutations (tld') rendering cells resistant to the killing effect of LetD protein is dominant over the wild type (LetD') allele (17). With respect to the inhibitory mechanism, the LetD-mediated gyrase inhibition creates an SOS signal (11); merely the elimination of gyrase activity is not sufficient for RecA induction (38). One of the tld' mutations causes an amino acid substitution at the 214th residue in the N-terminal domain of the GyrA protein (17), in which the nicking and closing activities reside (39). The LetD protein might interfere with some aspects of the nicking and closing events in the DNA supercoiling reaction, although other possibilities would need to be considered. As in the case of antigyrase drugs, the LetD protein as a protein inhibitor of the GyrA protein might provide a means to elucidate the yet-to-be elucidated mechanisms and structure-function relationships of DNA gyrase.

DNA gyrase plays a fundamental role in cellular processes, including DNA replication, transcription, recombination, as well as the resolution of daughter chromosomes (40, 41). These pleiotropic effects may be attributed to various enzymatic activities of the gyrase and also to the fact that most activities involved in the DNA transaction require a supercoiled state of DNA and a higher order structure of the chromosome in which the gyrase might be a structural component (42, 43). Consequently, a number of gyrase mutants so far isolated and cells treated with various inhibitors show different phenotypes depending on the manner in which gyrase is altered or affected (41). When the LetD protein functions to kill cells, the cells form filaments with large unseparated nucleoids irregularly clustered in the center of cells (4, 5). Some normal size anucleate cells are also generated by the aberrant division of the filaments (5). Within the same time span, DNA synthesis in these cells continues, albeit at a slightly reduced rate (5). Therefore, DNA gyrase in these cells seems to be impaired in a specific manner so as not to be able to conduct a proper segregation of the replicated nucleoids without a primary defect in DNA synthesis. Certain conditional lethal mutants carrying an amber mutation in the gyrA gene or temperature-sensitive mutations in the gyrB gene show essentially the same phenotype (13-15, 44). Under physiological circumstances, the LetD protein might preferentially inhibit the decatenation activity of DNA gyrase, which was shown to be required for the resolution of duplicated nucleoids (45). In addition, a perturbation of the topological state of DNA or the higher order structure of chromosone caused by the LetD protein might affect processes of chromosome segregation.

Although opposing actions of DNA gyrase and DNA topoisomerase I play a central role in determining the level of DNA supercoiling in bacterial cells (40, 46), accumulating evidence suggests that there are other proteins that influence DNA supercoiling. The hns (osmZ) gene product, an abundant DNA-binding protein (H-N-S), is one such example (47, 48). The topological changes brought about by the H-N-S protein may alter expression of a variety of genes dispersed throughout the E. coli genome (49, 50). It has also been suggested that HU (heterodimer of HU-1 and HU-2), a histone-like protein, and products of genes in the minB locus which are implied in the proper positioning of septa, are involved in modulation of DNA supercoiling. A reduced level of DNA supercoiling was found in a strain lacking both HU-1 and HU-2 or one with an unbalanced expression of genes in the minB locus (51-53). Interestingly, defective chromosome segregation and cell filamentation, the phenotype reminiscent of the letD-expressing cells, were observed with these strains (53-55). The gem gene of phage Mu encodes a protein that affects DNA supercoiling presumably through interaction with the B subunit of DNA gyrase (56, 57). The protein enables an efficient proliferation of the phage upon infection by altering expression of the host genes through effects on DNA supercoiling. In addition, a cyclical variation of gyrase activity or supercoiling state of DNA in the bacterial cell was suggested by the interesting features of Mu phage carrying a mutation in the gem gene (Mu gemt2) (58). The present study revealed that the LetD and LetA proteins are in this category. Among such proteins, the LetD and LetA are the only ones seen to have direct effects on DNA gyrase. In addition, the LetD and LetA proteins are unique in that they can reversibly modulate activity of the DNA gyrase. Although the killing effect of LetD protein has been considered to have biological importance (5), the rejuvenation capacity of LetA protein found in the present study suggests a different view concerning the LetD-mediated inactivation of DNA gyrase. It is feasible that the LetD and LetA proteins form an opposing pair biologically significant for modulation of the DNA gyrase. In this light, the roles of the LetA and LetD proteins during the steady-state growth of bacteria carrying the F plasmid might need to be reconsidered.

Acknowledgments—We thank M. Imai for generously providing plasmid, K. Sekimizu for GyrA, GyrB, and E. coli topoisomerase I proteins, and J. Kato for rabbit anti-GyrA serum. We also thank K. Nakasugi (Dai-ichi Seiyaku Co.) for helpful suggestions on plasmid constructions, K. Yamaguchi, H. Nakano (Kyowa Hakko Co.), T. Miyata, and F. Tokunaga for the amino acid analyses, and M. Ohara for reading the manuscript.

REFERENCES
1. Ogura, T., and Hiraqa, S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4784-4788
2. Bex, F., Karoui, H., Rokeach, L., Dréze, P., Garcia, L., and Couturier, M. (1983) EMBO J. 2, 1852-1861
3. Miki, T., Yoshioka, K., and Horiuchi, T. (1984) J. Mol. Biol. 174, 605-625
4. Miki, T., Chang, Z.-T., and Horiuchi, T. (1984) J. Mol. Biol. 174, 627-646
5. Jaffé, A., Ogura, T., and Hiraqa, S. (1985) J. Bacteriol. 163, 841-849
6. Hiraqa, S., Jaffé, A., Ogura, T., Mori, H., and Takahashi, H. (1986) J. Bacteriol. 166, 100-104
7. Karoui, H., Bex, F., Dréze, P., and Couturier, M. (1983) EMBO J. 2, 1863-1868
8. Brandenburger, A., Bailone, A., Lévine, A., and Devoret, R. (1984) J. Mol. Biol. 179, 571-576

T. Miki, unpublished results.
Modulation of DNA Gyrase by F Plasmid Proteins

12251

9. Mori, H., Ogura, T., and Hiraga, S. (1984) Mol. Gen. Genet. 216, 186-193
10. Sommer, S., Bailone, A., and Devoret, R. (1985) Mol. Gen. Genet. 218, 456-464
11. Bailone, A., Sommer, S., and Devoret, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5218-5222
12. Hirota, Y., Ricard, M., and Shapiro, B. (1971) Biomembranes 2, 13-31
13. Kato, J., Nishimura, Y., and Suzuki, H. (1989) Mol. Gen. Genet. 217, 175-181
14. Hussain, K., Elliott, E. J., and Salmond, G. P. C. (1987) Mol. Microbiol. 1, 259-275
15. Miki, T., Orita, T., Furuno, M., and Horichi, T. (1988) J. Mol. Biol. 201, 327-338
16. Miki, T., Park, J. A., Nagao, K., Murayama, N., and Horichi, T. (1992) J. Mol. Biol., in press
17. Miki, T., Yasukochi, T., Nagatani, H., Furuno, M., Orita, T., Yamada, H., Imoto, T., and Horichi, T. (1987) Protein Eng. 1, 327-332
18. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Cross, J. H., and Falkow, S. (1977) Gene (Amst.) 2, 95-115
19. Miki, T., Easton, A. M., and Rownd, R. H. (1978) Mol. Gen. Genet. 158, 217-224
20. Vieira, J., and Messing, J. (1982) Gene (Amst.) 19, 259-268
21. Maki, S., and Kornberg, A. (1977) J. Biol. Chem. 252, 680-685
22. Miki, T., Oritia, T., Furuno, M., and Horichi, T. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 73-81
23. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Gelbett, M., Mizzuchi, K., O'Dea, M. H., Ioh, T., and Tomizawa, J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 4772-4776
25. Pettigrew, D. W., Ma, D.-P., Conrad, C. A., and Johnson, J. R. (1988) J. Biol. Chem. 263, 135-159
26. Burnette, W. N. (1981) Anal. Biochem. 112, 195-203
27. Laemmli, U. K. (1970) Nature 227, 680-685
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
29. Sugino, A., Higgins, N. P., and Cozzarelli, N. R. (1980) Nucleic Acids Res. 8, 3865-3874
30. Wang, J. C. (1971) J. Mol. Biol. 55, 523-533
31. Ellis, R. J., and Hemingsen, S. M. (1988) Trends Biochem. Sci. 14, 339-342
32. Goloubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 342, 584-589
33. Tam, J. E., and Kline, B. C. (1989) Mol. Gen. Genet. 219, 26-32
34. Drlica, K., and Franco, R. J. (1988) Biochemistry 27, 2253-2259
35. Drlica, K., Engle, E. C., and Manes, S. H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6879-6883
36. Snyder, M., and Drlica, K. (1979) J. Mol. Biol. 131, 287-302
37. Staudenbauer, W. L. (1976) Eur. J. Biochem. 62, 491-497
38. Smith, C. L. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2510-2513
39. Reece, R. J., and Maxwell, A. (1991) J. Biol. Chem. 266, 3540-3546
40. Drlica, K. (1984) Microbiol. Rev. 48, 273-288
41. Vosberg, H.-P. (1985) Curr. Top. Microbiol. Immunol. 114, 19-102
42. Drlica, K. (1987) in Escherichia coli and Salmonella typhimurium (Neidhardt, F. C., ed) pp. 91-103, ASM Press, Washington, D. C.
43. Yang, Y., and Ames, G. F.-L. (1990) in The Bacterial Chromosome (Drlica, K., and Riley, M., eds) pp. 211-225, ASM Press, Washington, D. C.
44. Orr, E., Fairweather, N. F., Holland, I. B., and Pritchard, R. H. (1979) Mol. Gen. Genet. 177, 103-112
45. Steck, T. R., and Drlica, K. (1984) Cell 36, 1081-1088
46. Wang, J. C. (1985) Annu. Rev. Biochem. 54, 665-697
47. Hulton, C. S. J., Seirafi, A., Hinton, J. C. D., Sidebotham, J. M., Waddell, L., Pavitt, G. D., Owen-Hughes, T., Spassky, A., Buc, H., and Higgins, C. F. (1990) Cell 63, 631-642
48. May, G., Dersch, P., Haardt, M., Middendorf, A., and Bremer, E. (1990) Mol. Gen. Genet. 224, 81-90
49. Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G., and Bremer, E. (1998) Cell 52, 569-584
50. Higgins, C. F., Hinton, J. C. D., Hulton, C. S. J., Owen-Hughes, T., Pavitt, G. D., and Seirafi, A. (1990) Mol. Microbiol. 4, 2007-2012
51. Hillyard, D. R., Edlund, M., Hughes, K. T., Marsh, M., and Higgins, N. F. (1990) J. Bacteriol. 172, 5402-5407
52. Hsieh, L.-S., Rouviere-Yaniv, J., and Drlica, K. (1991) J. Bacteriol. 173, 3914-3917
53. Mulder, E., El'Bouhali, M., Pas, E., and Woldringh, C. L. (1990) Mol. Gen. Genet. 221, 87-93
54. Wada, M., Kano, Y., Ogawa, T., Okazaki, T., and Imamoto, F. (1988) J. Mol. Biol. 204, 581-591
55. Dri, A.-M., Rouviere-Yaniv, J., and Moreau, P. L. (1991) J. Bacteriol. 173, 2852-2863
56. Guelardini, P., Liebart, J. C., Marchelli, C., Pedrini, A. M., and Paozolli, L. (1984) J. Bacteriol. 157, 665-666
57. Ghelardini, P., Liebart, J. C., Paozolli, L., and Pedrini, A. M. (1989) Mol. Gen. Genet. 216, 31-36
58. Paozolli, L., Nicosia, A., Liebart, J. C., and Ghelardini, P. (1989) Mol. Gen. Genet. 218, 13-17