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Purification and Properties of DNA Gyrase from a Fluoroquinolone-Resistant Strain of *Escherichia coli*

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Subunit A and B proteins of DNA gyrase were separately purified from fluoroquinolone-resistant *Escherichia coli* GN14181 (MIC of ofloxacin, 100 μg/ml) and susceptible strain KL-16. The supercoiling activities of reconstituted Ar+Br (r, resistant) and Ar+Bs (s, susceptible) were 250-fold more resistant to new fluoroquinolones than those of As+Bs and As+Br.

DNA gyrase was discovered by Gellert et al. (8) as an activity in *Escherichia coli* that catalyzes the ATP-coupled negative supercoiling of DNA. In addition to supercoiling activity, the enzyme causes the relaxation of supercoiling DNA in the absence of ATP, and the binding of the enzyme to DNA leads to double-strand breakage at specific sites promoted by quinolones and sodium dodecyl sulfate (4, 5, 10, 17, 20). The enzyme activities result from breakage and rejoining of both strands of duplex DNA (3, 5). The active gyrase holoenzyme is a tetramer composed of two subunits, A_2 and B_2 (10, 15). It is believed that subunit A protein is the target of quinolones because nalidixic acid-resistant *gyrA* mutants are also resistant to quinolones (11, 12). New fluoroquinolones, such as ofloxacin, norfloxacin, ciprofloxacin, and enoxacin, have broad spectra against gram-negative and -positive bacteria (13, 14, 16, 19), and they strongly inhibit the supercoiling activity of DNA gyrase from *E. coli* KL-16 (18). However, there have been no reports on the inhibitory properties of DNA gyrase from clinical isolates.

In this report, we present the purification and properties of subunit A and B proteins of DNA gyrase from a fluoroquinolone-resistant isolate, *E. coli* GN14181.

E. coli GN14181 was isolated from an upper urinary tract infection and identified by the method of *Bergey's Manual of Systematic Bacteriology* (3). *E. coli* K-12 strains KL-16 (Hfr, *thi*, *relA*) and MH-5, its *gyrA*^{r} derivative, were also used (2, 9).

We received the following compounds as gifts from their manufacturers: ofloxacin (OFX), norfloxacin (NFX), ciprofloxacin (CPX), enoxacin (ENX), pipemidic acid, and nalidixic acid (NA). Novobiocin (NB) and coumermycin A1 (COU) were purchased from Sigma Chemical Co.

The agar dilution method was used to determine drug susceptibility. One loopful (ca. 2.5×10^4 CFU) of a diluted culture was inoculated onto modified Mueller-Hinton agar (Nissui Seiyaku Co., Ltd.). MICs were determined after incubation at 37°C for 18 h.

Crude enzyme was prepared by the method of Gellert et al. (6), loaded on an NB-Sepharose column (21), and eluted stepwise with 0.2 M NaCl–1 M NaCl–5 M urea in TGED buffer (50 mM Tris hydrochloride [pH 7.5], 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol). The A subunit was eluted with 1 M NaCl. The active fraction was further purified by using a heparin-Sepharose CL-6B (Pharmacia Fine Chemicals) column. Active fractions, which eluted around 0.3 M NaCl, were pooled after dialysis against TGED buffer. The B subunit was eluted with 5 M urea from an NB-Sepharose column.

The assay of supercoiling activity was modified from previous reports (6, 7). 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. (8). The reaction mixture containing subunit A and B proteins (each, 0.1 μg of protein), drug solution, and pBR322 relaxed by topoisomerase I (Bethesda Research Laboratories, Inc.) was incubated at 37°C for 1 h, and the reaction was stopped by the addition of proteinase K (20 μg/ml). The mixtures were subjected to 0.8% agarose gel electrophoresis. The gel was stained with ethidium bromide (0.5 μg/ml) and photographed by using UV light. The negatives were traced with a densitometer. The concentration of protein was estimated by measuring the A_{280}.

The antimicrobial susceptibility of the *E. coli* strains is shown in Table 1. *E. coli* GN14181 was highly resistant to OFX, NFX, and ENX. In contrast, KL-16 was inhibited at low concentrations of these drugs. The *gyrA* mutant, MH-5, demonstrated an intermediate degree of resistance.

Subunit A and B proteins were separately purified from a crude extract of strain GN14181. The specific activities of the purified A and B subunits were about 10^4 U/mg of protein. The enzyme activity of the crude extract could not be measured, because DNase in the crude extract decomposed DNA as substrate. The purities of both A and B subunits were about 90% on sodium dodecyl sulfate-

TABLE 1. Susceptibility of *E. coli* GN14181, KL-16, and MH-5 (*gyrA*^{r}) to quinolones and NB

Antimicrobial agent	MIC (μg/ml) in *E. coli*		
	GN14181	KL-16	MH-5
OFX	100	0.05	1.56
NFX	100	0.05	1.56
CPX	25	≤0.006	0.39
ENX	100	0.10	3.13
PPA[a]	>400	1.56	50
NA	>400	6.25	>400
NB	100	50	50

[a] PPA, Pipemidic acid.

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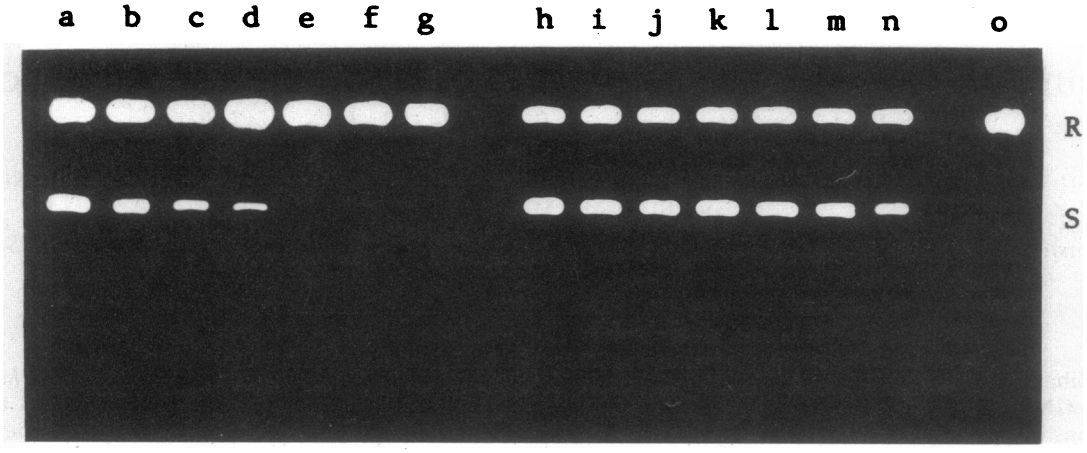

FIG. 1. Comparative inhibitory effects of OFX on supercoiling activities of reconstituted subunit A and B proteins from *E. coli* GN14181 and subunit A and B proteins from KL-16. Relaxed (R) and supercoiled (S) pBR322 was separated on a 0.8% agarose gel. Lane A, Relaxed DNA with 1 U of subunit A (0.1 μg of protein) and subunit B (0.1 μg of protein) from KL-16. Lanes b to g were the same as lane a with OFX at the following concentrations: b, 1.0 μg/ml; c, 2.0 μg/ml; d, 3.9 μg/ml; e, 7.8 μg/ml; f, 15.6 μg/ml; and g, 31.3 μg/ml. Lane h, Relaxed DNA with 1 U of subunit A (0.1 μg/ml of protein) plus subunit B (0.1 μg/ml of protein) from GN14181. Lanes i to n were the same as lane h with OFX at the following concentrations: i, 25 μg/ml; j, 50 μg/ml; k, 100 μg/ml; l, 200 μg/ml; m, 400 μg/ml; and n, 800 μg/ml. Lane o, Relaxed DNA alone. Supercoiling assay conditions were used except in lane o.

polyacrylamide gel electrophoresis. The A and B subunits were also purified from strains KL-16 and MH-5. The major protein bands of A subunits from GN14181 (Ar) (r, resistant), KL-16 (As) (s, susceptible), and MH-5 (Am) (m, moderately resistant) were 110,000, 105,000, and 110,000, respectively. The B subunits (Br, Bs, and Bm) had molecular weights of 95,000.

The supercoiling activities of As+Bs and Ar+Br are shown in Fig. 1. The assay conditions were defined to measure the point at which 50% of relaxed DNA was converted to supercoiled form. Lanes b to g were the same as lane a with OFX at various concentrations. OFX blocked the As+Bs activity; some inhibition was seen at 2 μg/ml (lane c), and complete inhibition occurred at 7.8 μg/ml (lane e). In contrast, lanes i to n were the same as lane h with OFX at high concentrations. Ar+Br was resistant to 800 μg of OFX per ml. The inhibitory patterns of OFX, NFX, CPX, and ENX at various concentrations against the activities of As+Bs and Ar+Br are shown in Fig. 2. Ar+Br was highly resistant to the new agents. Furthermore, the mixture of Ar+Bs was also resistant to the drugs (Table 2). The 50% inhibition doses of the new agents against As+Bs and As+Br ranged from 0.8 to 11.2 μg/ml. However, the 50% inhibition doses of the drugs were 800 μg/ml or greater against Ar+Br and Ar+Bs. Am+Bs was intermediately resistant to the drugs. The 50% inhibition doses of NB and

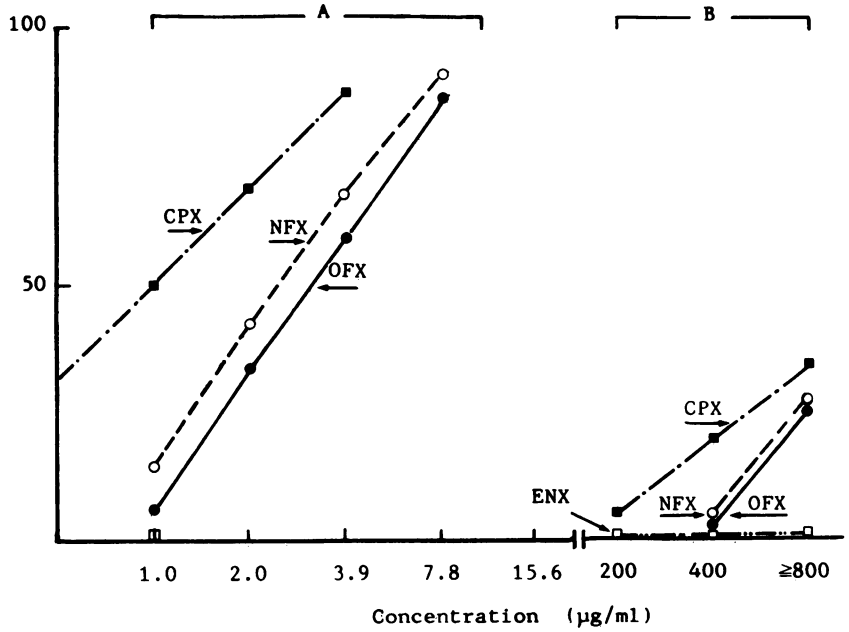

FIG. 2. Inhibitory patterns of new fluoroquinolones against activities of reconstituted subunit A and B proteins of *E. coli* KL-16 (A) and GN14181 (B). The inhibitory percentage was calculated by densitometer tracing.

TABLE 2. Inhibitory concentrations of quinolones, NB, and COU on supercoiling activity of reconstituted DNA gyrase

Antimicrobial agent	50% Inhibition dose (μg/ml)[a] for reconstituted DNA gyrase of[b]:					
	Ar + Br	Ar + Bs	As + Bs	As + Br	Am + Bm	Am + Bs
OFX	>800	>800	3.1	2.8	150	152
NFX	>800	>800	2.4	2.4	225	220
CPX	>800	>800	1.0	0.8	112	110
ENX	>800	>800	11.2	8.4	>400	>400
PPA[c]	>800	>800	156	140	>400	>400
NA	>800	>800	>400	>400	>400	>400
NB	≤0.10	≤0.10	≤0.10	≤0.10	≤0.10	≤0.10
COU	≤0.10	≤0.10	≤0.10	≤0.10	≤0.10	≤0.10

[a] Calculated by measuring the supercoiled DNA peak by densitometry.
[b] Ar, A subunit from GN14181; Br, B subunit from GN14181; As, A subunit from KL-16; Bs, B subunit from KL-16; Am, A subunit from MH-5; Bm, B subunit from MH-5.
[c] PPA, Pipemidic acid.

COU were less than 1.0 μg/ml against each of the gyrase mixtures.

NA-resistant mechanisms of *E. coli* were reported to involve mutations in *gyrA* (formerly *nalA*), *gyrB*, (*cou*, *nalC*), or *ompF* genes (1, 2, 11, 23). Cross resistance is usually observed between NA and the new related drugs but may be incomplete (11, 12, 23). This study also showed that the MICs of OFX, NFX, CPX, and ENX were 32 times lower than that of NA against strain MH-5. However, the new agents could not inhibit the bacterial growth of strain GN14181 at high concentrations. Resistance to new fluoroquinolones, such as NFX (MIC, 100 μg/ml), at so high a level has not been attained even with serial passage of *E. coli* on increasing drug concentrations in the laboratory (11, 22).

The supercoiling activity of DNA gyrase from strain GN14181 was highly resistant to the new quinolones. Furthermore, the reconstitution of the A subunit from GN14181 and the B subunit from KL-16 was also resistant. In contrast, the mixture of the A subunit from KL-16 and the B subunit from GN14181 was sensitive to the new agents. There was a parallel relationship between the inhibitory effects of the new agents on the supercoiling activities of the subunit A proteins from GN14181, MH-5, and KL-16 plus the B subunit from KL-16 and the antibacterial activities of the drugs. However, the drug concentrations for the inhibition of supercoiling activity were 100-fold higher than the MICs. Gellert et al. (6) and Yamagishi et al. (23) reported similar results. These results suggested that bacterial cells must be very susceptible to rather small changes in DNA supercoiling.

On the basis of these results, we conclude that one of the resistance mechanisms of *E. coli* GN14181 against new fluoroquinolones is a modification of the subunit A protein, and our study also supports the role of the gyrase A subunit as the active site for quinolones.

Since such a highly resistant A subunit of *E. coli* was not previously described, we plan further enzymological studies on, e.g., DNA breakage in the presence of new fluoroquinolones and the genetic bases of this phenomenon.

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