ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1993, p. 839–845
0066-4804/93/040839-07$02.00/0

Vol. 37, No. 4

# Mechanism of Action of Quinolones against *Escherichia coli* DNA Gyrase

HIROAKI YOSHIDA,* MIKA NAKAMURA, MAYUMI BOGAKI, HIDEAKI ITO, TSUYOSHI KOJIMA, HIROAKI HATTORI, AND SHINICHI NAKAMURA

*Bioscience Research Laboratories, Dainippon Pharmaceutical Co., Ltd., Enoki 33-94, Suita, Osaka 564, Japan*

Received 27 July 1992/Accepted 27 January 1993

**The mechanism of action of quinolones was investigated by use of various DNA gyrases reconstituted from wild-type and mutant GyrA and GyrB proteins of *Escherichia coli*. The quinolone sensitivities of the DNA supercoiling activity of the gyrases were generally parallel to the quinolone susceptibilities of strains having the corresponding enzymes and depended on gyrase subunits but not on substrate DNA. [$^3$H]Enoxacin did not bind to gyrase alone or DNA alone but bound to gyrase-DNA complexes when measured by a gel filtration method. There appeared to be two enoxacin binding phases, at low and high enoxacin concentrations, for the wild-type gyrase-DNA and type 2 GyrB (Lys-447 to Glu) mutant gyrase-DNA complexes but only one enoxacin binding phase at the concentrations used for the GyrA (Ser-83 to Leu) mutant gyrase-DNA and type 1 GyrB (Asp-426 to Asn) mutant gyrase-DNA complexes. New enoxacin binding sites appeared in the presence of enoxacin, and the enoxacin binding affinities for the sites, especially at low enoxacin concentrations, near the MICs for the strains having the corresponding gyrases, correlated well with the enoxacin sensitivities of the gyrases and the MICs. From the results obtained, we propose a quinolone pocket model as the mechanism of action of quinolones, in which quinolones exert their action through binding to a gyrase-DNA complex and the quinolone binding affinities for the complex are determined by both GyrA and GyrB subunits in concert.**

Quinolones are a group of antibacterial agents currently used for various kinds of infections. The molecular target of quinolones is considered to be DNA gyrase (1, 25), since quinolones inhibit gyrase activities and gyrases isolated from quinolone-resistant strains are resistant to quinolones (3, 19, 23). *Escherichia coli* gyrase (EC 5.99.1.3) consists of subunits A and B, which are the products of the *gyrA* and *gyrB* genes, respectively (6, 9, 16). Since the unexpected finding by Shen and Pernet that [$^3$H]norfloxacin binds to DNA but not to purified gyrase (22), it has been proposed that quinolones may inhibit gyrase activity through binding to DNA. This proposal still exists after the later finding that a new quinolone binding site appears upon the formation of gyrase-DNA complexes and that quinolone binding to the site is closely correlated with inhibition of gyrase activity (20). On the basis of such binding data, a cooperative quinolone-DNA binding model has been proposed, in which a quinolone binds to bases of single-stranded DNA and self-associates in a pocket created by gyrase (21). However, this model cannot explain why mutational changes in the gyrase genes can cause quinolone resistance (27–30). To learn more about the mechanism of action of quinolones, we examined the quinolone sensitivities of gyrases reconstituted from purified wild-type and mutant GyrA and GyrB proteins and [$^3$H] enoxacin binding to gyrase-DNA complexes.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* KL16 was kindly provided by Barbara Bachmann of Yale University, and quinolone-resistant mutants of KL16 (GyrA mutant, N-51; type 1 GyrB mutant, N-24; and type 2 GyrB mutant, N-31) were isolated as described previously (27, 30). Plasmids pBR322 and pHY300PLK were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Plasmid pHTP318 (26) was provided by Junichi Yamagishi, Bioscience Research Laboratories, Dainippon Pharmaceutical Co., Ltd. The derivation of plasmids pAW012, containing the wild-type *E. coli gyrA* gene (17), and pJB11, containing the wild-type *E. coli gyrB* gene (27), and plasmids carrying mutant *gyrA* or *gyrB* genes was described previously (27, 30).

**Reagents.** Nalidixic acid (11), oxolinic acid (8), enoxacin (12), and sparfloxacin (14) were synthesized at Exploratory Research Laboratories, Dainippon Pharmaceutical Co. Restriction endonucleases, T4 DNA ligase, and a bicinchoninic acid protein assay kit were purchased from Takara Shuzo Co. DEAE–Sepharose CL-6B, heparin–Sepharose CL-6B, and NICK columns (Sephadex G-50) were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Bio-Gel HTP (hydroxylapatite) was purchased from Bio-Rad Laboratories (Richmond, Calif.). [$^3$H]Enoxacin was synthesized by Amersham International (Buckinghamshire, United Kingdom) by means of a process developed at Developmental Research Laboratories, Dainippon Pharmaceutical Co. In brief, [$^3$H]enoxacin was prepared by hydrogenation with $^3H_2$ of 6-fluoro-7-piperazinyl-1-vinyl-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid. This labeled enoxacin had a specific activity of 16.6 Ci/mmol and was diluted appropriately with cold enoxacin. (*p*-Amidinophenyl)methanesulfonyl fluoride (*p*-APMSF) was purchased from Wako Chemicals (Osaka, Japan). Other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

**Preparation of plasmid DNA.** Small-scale plasmid DNA isolation was carried out by the rapid boiling method described by Holmes and Quigley (7).

**Transformation.** Transformation was performed by the $CaCl_2$ method, and the transformants were selected on LB agar (13) containing ampicillin at 25 μg/ml.

**Production of GyrA and GyrB proteins.** As shown in Fig. 1, the 1,092-bp *Pst*I-*Dra*I DNA fragment of pHTP318 was used

* Corresponding author.

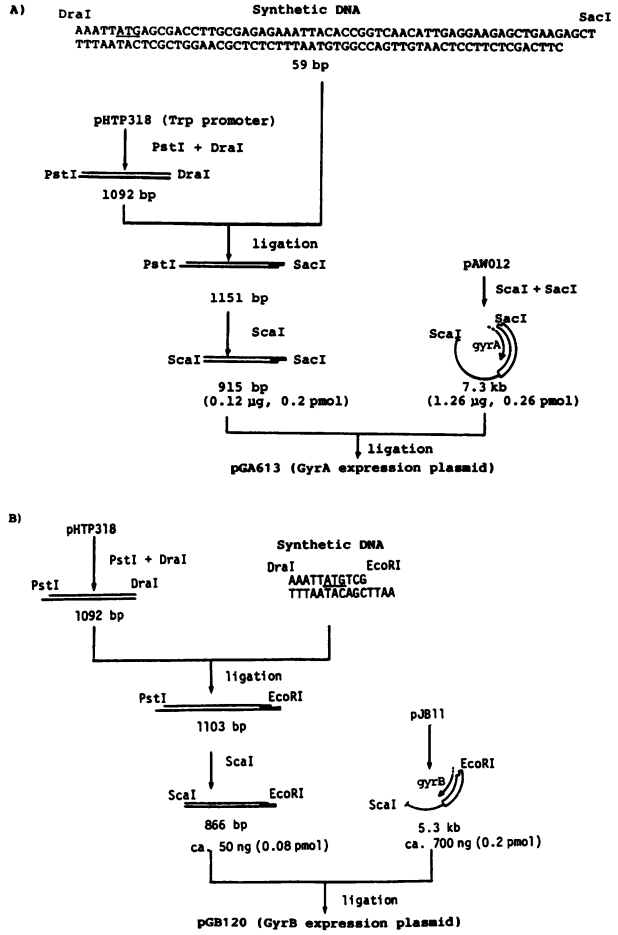


FIG. 1. Construction of the expression plasmids for the wild-type *E. coli* GyrA (A) and GyrB (B) proteins.

as the source of the *E. coli trp* promoter. For the construction of a GyrA expression plasmid, this fragment was ligated with a 59-bp synthetic oligonucleotide; ligation was followed by cutting with *Sca*I to generate a 915-bp *Sca*I-*Sac*I DNA fragment (Fig. 1A). GyrA expression plasmid pGA613 was produced by ligation of the 915-bp DNA fragment with a 7.3-kb *Sca*I-*Sac*I fragment of pAW012 into which the *E. coli* wild-type *gyrA* gene had been cloned. GyrB expression plasmid pGB120 was constructed in a similar way (Fig. 1B). Expression plasmids for mutant GyrA and GyrB production were constructed by means of the same strategy. Isogenic *E. coli* strains were transformed with one of these plasmids, and the GyrA and GyrB proteins were produced by cultivating the transformants in LB broth at 37°C for 20 h with 3β-indoleacrylic acid (20 μg/ml) for derepression of the *gyrA* and *gyrB* genes under the control of the *trp* promoter.

**Purification of GyrA and GyrB proteins.** All purification steps were carried out at 4°C. *p*-APMSF was added at a concentration of 1 mM to all the solutions to prevent proteolysis. For fractionation, the GyrA and GyrB proteins were monitored by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. For the purification of GyrA, a cleared lysate was obtained from 0.5 to 2 liters of culture by the method of Gellert et al. (2). Nucleic acids were removed by centrifugation at 15,000 × *g* for 10 min after the addition of streptomycin (5% [wt/vol]). To the supernatant was added ammonium sulfate at a concentration of 0.31 g/ml. After the mixture was allowed to stand for 30 min on ice, it was centrifuged at 15,000 × *g* for 10 min, and the precipitate was dissolved in 20 ml of TGED buffer (50 mM Tris-HCl [pH 7.5], 10% [wt/vol] glycerol, 1 mM $Na_3EDTA$, 5 mM dithiothreitol). The solution was dialyzed against 5 liters of TGED buffer for 4 h. The dialysate was rapidly applied to a column (2.6 by 7 cm) of DEAE–Sepharose CL-6B and eluted with 300 ml of TGED buffer containing a linear NaCl concentration gradient (0 to 0.7 M). Fractions containing GyrA were dialyzed as described above and then applied to a heparin–Sepharose CL-6B column (2.6 by 5 cm) and eluted with 300 ml of TGED buffer containing a linear NaCl concentration gradient (0 to 0.5 M). For the purification of GyrB, the same procedures were used, except that GyrB was solubilized with 6 M urea at 30°C for 20 min just before the first centrifugation. The dialysate obtained after ammonium sulfate precipitation was applied to a heparin–Sepharose CL-6B column (2.6 by 7 cm) and eluted with 300 ml of TGED buffer containing a linear NaCl concentration gradient (0 to 0.7 M). Fractions containing GyrB were pooled, dialyzed for 3 h, and then applied to a hydroxylapatite column (1 by 7 cm). Elution was carried out with 300 ml of a linear potassium phosphate (pH 6.8) concentration gradient (0.02 to 0.5 M).

**Assay for supercoiling activities of gyrases.** Gyrases were reconstituted from various purified GyrA and GyrB proteins (20 U each) in a volume of 20 μl. Their supercoiling activities were measured by the method of Sato et al. (19) with pBR322 and pHY300PLK DNAs as substrates. One unit was defined as the amount of enzyme that catalyzed the conversion of 50% of relaxed DNA to a supercoiled form in 1 h at 30°C. The specific activity of GyrA (or GyrB) was determined in the presence of 20 U of its counterpart.

**Determination of protein concentrations.** Protein concentrations were determined with the bicinchoninic acid protein assay kit in accordance with the manufacturer's instructions and with bovine serum albumin as a standard.

**Measurement of [$^3$H]enoxacin bound to gyrase-DNA complexes.** The incubation conditions were essentially the same as those of Shen et al. (20). The reaction mixture contained, in 50 μl of 50 mM Tris-HCl (pH 7.5), various concentrations of GyrA and GyrB (1 to 30 pmol each), *Eco*RI-cleaved pBR322 DNA (0.005 to 8 pmol), 1 mM dithiothreitol, 6 mM $MgCl_2$, 20 mM KCl, and 1 mM ATP. This mixture was preincubated at 30°C for 60 min, and then [$^3$H]enoxacin (1 to 1,000 pmol) was added. After further incubation for 120 min at 30°C, the mixture was applied to a NICK column (Sephadex G-50; 0.9 by 2 cm); radioactivity recovered from the macromolecule fraction was measured by scintillation counting, from which the amounts of [$^3$H]enoxacin bound to gyrase-DNA complexes were calculated.

## RESULTS

**Preparation and characterization of the GyrA and GyrB proteins.** The quinolone susceptibilities and gyrase mutations of *E. coli* KL16 derivatives from which the *gyrA* and *gyrB* genes were cloned are shown in Table 1. Compared with wild-type strain KL16, *gyrA* mutant N-51 and type 1 *gyrB* mutant N-24 were resistant to all the quinolones tested, but type 2 *gyrB* mutant N-31 was resistant to acidic quinolones, such as nalidixic and oxolinic acids, and concurrently hypersusceptible to amphoteric quinolones, such as enoxacin and sparfloxacin.

GyrA proteins $A^{KL16}$ and $A^{N51}$ were produced through expression of the *gyrA* genes cloned from KL16 and N-51,

TABLE 1. *E. coli* KL16 derivatives from which gyrase genes were cloned[a]

| Strain | MIC (μg/ml) of: | | | | Mutation |
|---|---|---|---|---|---|
| | Nalidixic acid | Oxolinic acid | Enoxacin | Sparfloxacin | |
| KL16 | 3.13 | 0.39 | 0.1 | 0.0125 | None (wild type) |
| N-51 | 400 | 6.25 | 1.56 | 0.2 | *gyrA*; Ser-83 (TCG)→Leu (TTG) |
| N-24 | 50 | 1.56 | 0.78 | 0.05 | Type 1 *gyrB*; Asp-426 (GAC)→Asn (AAC) |
| N-31 | 50 | 1.56 | 0.025 | 0.0031 | Type 2 *gyrB*; Lys-447 (AAG)→Glu (GAG) |

[a] Some of the data are from references 28 to 30.

respectively, and GyrB proteins $B^{KL16}$, $B^{N24}$, and $B^{N31}$ were produced through expression of the *gyrB* genes cloned from KL16, N-24, and N-31, respectively, in syngeneic strains. The proteins were purified to homogeneity, as judged by SDS-polyacrylamide gel electrophoresis (Fig. 2). The methionine residue corresponding to the initiation codon was found by N-terminal amino acid analysis to be removed in all the proteins.

The specific supercoiling activities of the GyrA and GyrB proteins, determined in the presence of an excess of the counterpart, are shown in Table 2. There were no marked differences between the wild-type and mutant GyrA proteins or between the wild-type and mutant GyrB proteins, indicating that the GyrA and GyrB mutations themselves did not markedly reduce the activities of the gyrases. On the other hand, there were marked differences between the GyrA and GyrB proteins, the specific activities of the latter being 1 or 2 orders of magnitude lower than those of the former. As gyrases consist of equimolar amounts of GyrA and GyrB proteins (6, 9, 16), this result suggests that our GyrB preparations contained an inactive form, irrespective of their homogeneity in SDS-polyacrylamide gel electrophoresis.

**Quinolone sensitivities of reconstituted gyrases.** The quinolone sensitivities of reconstituted wild-type and mutant gyrases were examined by means of a supercoiling reaction with relaxed pBR322 and pHY300PLK DNAs as substrates. The 50% inhibitory doses ($ID_{50}$s) of nalidixic acid, oxolinic acid, enoxacin, and sparfloxacin were not significantly different between these two plasmid DNAs for any gyrase, indicating that the DNA species had nothing in particular to do with the quinolone sensitivity (Table 3).

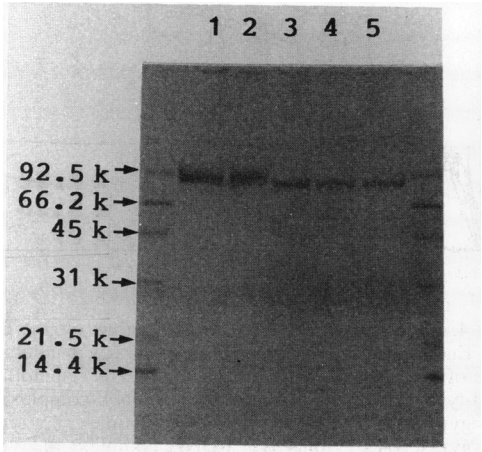


FIG. 2. SDS-polyacrylamide gel electrophoretogram of the purified GyrA and GyrB proteins of *E. coli*. About 1 to 2 μg of each protein was subjected to SDS-polyacrylamide gel electrophoresis. Lanes: 1, $A^{KL16}$; 2, $A^{N51}$; 3, $B^{KL16}$; 4, $B^{N24}$; 5, $B^{N31}$.

The GyrA mutant gyrase ($A^{N51}$ plus $B^{KL16}$) and the type 1 GyrB mutant gyrase ($A^{KL16}$ plus $B^{N24}$) were resistant to all the quinolones tested, when compared with the wild-type gyrase ($A^{KL16}$ plus $B^{KL16}$). The type 2 GyrB mutant gyrase ($A^{KL16}$ plus $B^{N31}$) was resistant to the acidic quinolones but hypersensitive to the amphoteric ones. These results demonstrate that the quinolone sensitivities of the gyrases are correlated with the quinolone susceptibilities of strains having the corresponding enzymes.

The GyrA-type 1 GyrB double-mutant gyrase ($A^{N51}$ plus $B^{N24}$) was as resistant to oxolinic acid and sparfloxacin as and more resistant to enoxacin than the respective single-mutant gyrases. The GyrA-type 2 GyrB double-mutant gyrase ($A^{N51}$ plus $B^{N31}$) was more resistant to oxolinic acid than each single-mutant gyrase but almost as sensitive to amphoteric quinolones as the wild-type gyrase. These results indicate that the quinolone sensitivities of gyrases are determined by both GyrA and GyrB subunits in concert and also depend on the chemical structures of the quinolones tested.

**Binding of [$^3$H]enoxacin to gyrase-DNA complexes.** The amounts of [$^3$H]enoxacin bound to the gyrase-DNA complexes were determined by gel filtration. Under the experimental conditions used, enoxacin did not bind to the wild-type gyrase alone or to DNA alone (or did bind only to a small extent) but did bind to gyrase-DNA complexes to a larger extent (Fig. 3). With 0.1 pmol of DNA, increasing amounts of the wild-type gyrase, and 250 pmol of enoxacin per 50 μl of reaction mixture, the amount of enoxacin bound to the gyrase-DNA complex reached a plateau of 0.84 pmol at gyrase concentrations higher than 11 pmol (Fig. 3A). With increasing amounts of DNA, 5.5 pmol of the wild-type gyrase, and 250 pmol of enoxacin per 50 μl of reaction mixture, the amount of enoxacin bound to the complex attained a plateau of 0.5 pmol at DNA concentrations higher than 0.05 pmol (Fig. 3B).

With 1 pmol of DNA, 5.5 pmol of various gyrases, and increasing amounts (1 to 1,000 pmol) of enoxacin per 50 μl of

TABLE 2. Specific activities of the purified *E. coli* gyrase subunits

| Gyrase subunit | Gene derivation | Mutation | Sp act (U/mg) |
|---|---|---|---|
| $A^{KL16}$ | KL16 | None (wild type) | $2.0 \times 10^6$ |
| $A^{N51}$ | N-51 | Ser-83→Leu | $3.6 \times 10^6$ |
| $B^{KL16}$ | KL16 | None (wild type) | $8.5 \times 10^4$ |
| $B^{N24}$ | N-24 | Asp-426→Asn | $2.3 \times 10^4$ |
| $B^{N31}$ | N-31 | Lys-447→Glu | $3.6 \times 10^4$ |

TABLE 3. Quinolone sensitivities of wild-type and mutant gyrases of *E. coli* in the DNA supercoiling reaction

| Gyrase | Gyrase subunits | $ID_{50}$, in μg/ml, of the following drug for DNA supercoiling[a]: | | | |
|---|---|---|---|---|---|
| | | Nalidixic acid | Oxolinic acid | Enoxacin | Sparfloxacin |
| Wild type | $A^{KL16} + B^{KL16}$ | 50 (100) | 3.13 (6.25) | 3.13 (6.25) | 0.39 (0.78) |
| GyrA mutant | $A^{N51} + B^{KL16}$ | >200 (>200) | 50 (100) | 25 (25) | 3.13 (6.25) |
| Type 1 GyrB mutant | $A^{KL16} + B^{N24}$ | >200 (>200) | 100 (100) | 25 (50) | 3.13 (6.25) |
| Type 2 GyrB mutant | $A^{KL16} + B^{N31}$ | >200 (>200) | 50 (50) | 1.56 (3.13) | 0.2 (0.2) |
| GyrA-type 1 GyrB double mutant | $A^{N51} + B^{N24}$ | >200 | 100 | 100 | 3.13 |
| GyrA-type 2 GyrB double mutant | $A^{N51} + B^{N31}$ | >200 | >200 | 6.25 | 0.39 |

[a] pBR322 or pHY300PLK (in parentheses) DNA was used as a substrate.

reaction mixture, there appeared to be two binding phases for the wild-type gyrase-DNA and the type 2 GyrB mutant gyrase-DNA complexes and only one binding phase for the GyrA mutant gyrase-DNA and the type 1 GyrB mutant gyrase-DNA complexes (Fig. 4). With the wild-type gyrase-DNA complex, the first binding phase was observed in the concentration range of 0.1 to 1 μM (5 to 50 pmol per 50 μl, or 0.032 to 0.32 μg/ml), close to the MIC (0.1 μg/ml) of enoxacin for wild-type strain KL16, and the second binding phase was observed at concentrations higher than 4 μM (200 pmol per 50 μl, or 1.3 μg/ml), close to the $ID_{50}$ (3.13 μg/ml) of enoxacin in the DNA supercoiling reaction for the wild-type gyrase. This also appeared to be the case with the type 2 GyrB mutant gyrase-DNA complex: the first binding phase was observed in the concentration range of 0.02 to 0.5 μM (1 to 25 pmol per 50 μl, or 0.0064 to 0.16 μg/ml), close to the MIC (0.025 μg/ml) of enoxacin for type 2 *gyrB* mutant N-31, and the second binding phase was observed at concentrations higher than 4 μM (200 pmol per 50 μl, or 1.3 μg/ml), close to the $ID_{50}$ (1.56 μg/ml) of enoxacin for the type 2 GyrB mutant gyrase. In contrast, enoxacin bound to the GyrA mutant gyrase-DNA complex with a single binding phase in the concentration range of 0.1 to 20 μM (5 to 1,000 pmol per 50 μl, or 0.032 to 6.4 μg/ml), including the MIC (1.56 μg/ml) of enoxacin for *gyrA* mutant N-51 but not the $ID_{50}$ (25 μg/ml) of enoxacin for the GyrA mutant gyrase. The total amount of

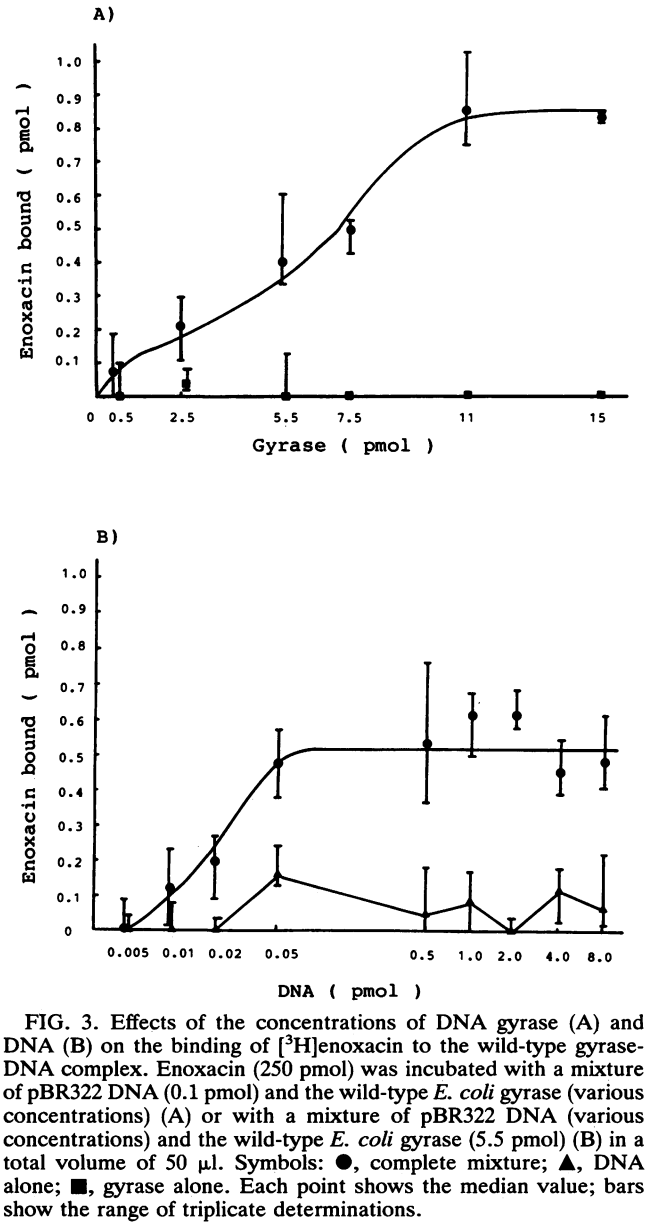


FIG. 3. Effects of the concentrations of DNA gyrase (A) and DNA (B) on the binding of [$^3$H]enoxacin to the wild-type gyrase-DNA complex. Enoxacin (250 pmol) was incubated with a mixture of pBR322 DNA (0.1 pmol) and the wild-type *E. coli* gyrase (various concentrations) (A) or with a mixture of pBR322 DNA (various concentrations) and the wild-type *E. coli* gyrase (5.5 pmol) (B) in a total volume of 50 μl. Symbols: ●, complete mixture; ▲, DNA alone; ■, gyrase alone. Each point shows the median value; bars show the range of triplicate determinations.

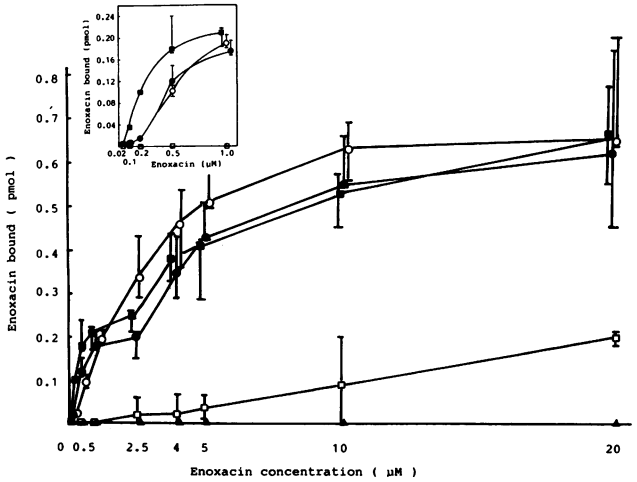


FIG. 4. Binding of [$^3$H]enoxacin to various gyrase-DNA complexes. Enoxacin at increasing concentrations was incubated with a mixture of gyrase (5.5 pmol) and pBR322 DNA (1 pmol). Symbols: ●, wild-type gyrase ($A^{KL16}$ plus $B^{KL16}$)-DNA complex; ○, GyrA mutant gyrase ($A^{N51}$ plus $B^{KL16}$)-DNA complex; □, type 1 GyrB mutant gyrase ($A^{KL16}$ plus $B^{N24}$)-DNA complex; ■, type 2 GyrB mutant gyrase ($A^{KL16}$ plus $B^{N31}$)-DNA complex; ▲, DNA alone. Each point shows the median value; bars show the range of triplicate determinations. The inset shows binding at concentrations of 0.02 to 1.0 μM [$^3$H]enoxacin.

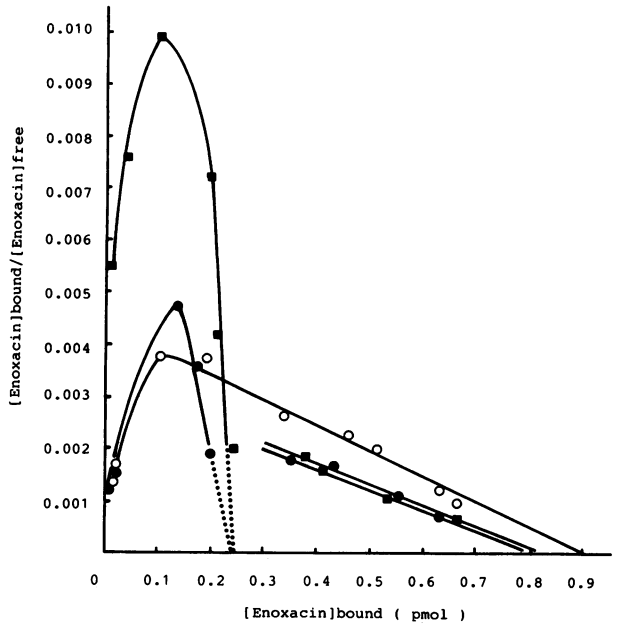


FIG. 5. Scatchard plots of [$^3$H]enoxacin binding to various gyrase-DNA complexes. Symbols: ●, wild-type gyrase ($A^{KL16}$ plus $B^{KL16}$)-DNA complex; ○, GyrA mutant gyrase ($A^{N51}$ plus $B^{KL16}$)-DNA complex; ■, type 2 GyrB mutant gyrase ($A^{KL16}$ plus $B^{N31}$)-DNA complex.

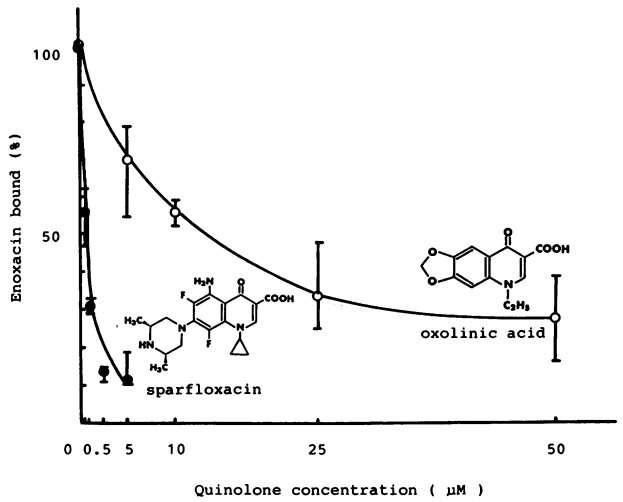


FIG. 6. Inhibition of the binding of [$^3$H]enoxacin to the wild-type gyrase-DNA complex by quinolones. Enoxacin (250 pmol) was incubated with pBR322 DNA (1 pmol) and gyrase (5.5 pmol) in a 50-μl reaction mixture in the presence of oxolinic acid (○) or sparfloxacin (●) at increasing concentrations. Each point shows the median value; bars show the range of triplicate determinations.

enoxacin bound to the GyrA mutant gyrase-DNA complex was similar to that bound to the wild-type gyrase-DNA or the type 2 GyrB mutant gyrase-DNA complex. Single-phase enoxacin binding was also observed with the type 1 GyrB mutant gyrase-DNA complex in the concentration range of 1 to 20 μM (50 to 1,000 pmol per 50 μl, or 0.32 to 6.4 μg/ml), including the MIC (0.78 μg/ml) of enoxacin for type 1 *gyrB* mutant N-24 but not the $ID_{50}$ (25 μg/ml) of enoxacin for the type 1 GyrB mutant gyrase, although the total amount of enoxacin bound to the complex was much smaller than that bound to the other three complexes. The enoxacin binding pattern of the GyrA-type 1 GyrB double-mutant gyrase-DNA complex was similar to that of the type 1 GyrB mutant gyrase-DNA complex, and the enoxacin binding pattern of the GyrA-type 2 GyrB double-mutant gyrase-DNA complex was similar to that of the type 2 GyrB mutant gyrase-DNA complex (data not shown). These results suggest that both GyrA and GyrB proteins play an important role in enoxacin binding to gyrase-DNA complexes.

Figure 5 shows Scatchard plots for the three gyrase-DNA complexes to which enoxacin bound sufficiently for analysis. The bound enoxacin/free enoxacin ratios increased up to the lower level of enoxacin bound of about 0.15 pmol, a result suggesting that enoxacin binding sites would appear in the presence of enoxacin. The ratios then decreased hyperbolically or linearly with increasing levels of enoxacin binding, a result suggesting that there were two kinds of site for enoxacin binding, with high and low affinities, in the wild-type gyrase-DNA and the type 2 GyrB mutant gyrase-DNA complexes and a single kind of site in the GyrA mutant gyrase-DNA complex. The dissociation constants ($K_d$s) of enoxacin at the high- and low-affinity binding sites for the wild-type gyrase-DNA complex were calculated to be 0.4 and 6 μM, respectively, and those for the type 2 GyrB mutant gyrase-DNA complex were calculated to be 0.1 and 6 μM, respectively. The amounts of enoxacin bound to the high- and low-affinity binding sites were calculated to be 0.24 and 0.8 pmol for both of the complexes. The $K_d$ of enoxacin for the GyrA mutant gyrase-DNA complex was calculated to be 4 μM, and the total amount of enoxacin bound was calculated to be 0.9 pmol. These results demonstrate that the $K_d$s of enoxacin for the gyrase-DNA complexes, especially at concentrations close to the MICs, when two sites exist are well correlated with the quinolone sensitivities of the gyrases and with the MICs for strains having the corresponding enzymes.

Enoxacin binding sharply decreased, with an inhibition constant ($K_i$) of 0.04 μM, when sparfloxacin was added in increasing concentrations to a 50-μl reaction mixture containing 1 pmol of DNA, 5.5 pmol of the wild-type gyrase, and 250 pmol of enoxacin and moderately decreased, with a $K_i$ of 0.9 μM, when oxolinic acid was added in increasing concentrations, results suggesting that quinolones specifically inhibit enoxacin binding, depending on their chemical structures (Fig. 6).

## DISCUSSION

All of the wild-type and mutant GyrA and GyrB proteins were produced through the expression of cloned genes whose sequences had been elucidated previously (27–30). Therefore, the mutant GyrA and GyrB proteins used have a point mutation: $A^{N51}$ (Ser-83 to Leu), $B^{N24}$ (Asp-426 to Asn), and $B^{N31}$ (Lys-447 to Glu). The specific activities of $A^{KL16}$ and $A^{N51}$ were $2 \times 10^6$ to $3.6 \times 10^6$ U/mg of protein, while those of $B^{KL16}$, $B^{N24}$, and $B^{N31}$ were $2.3 \times 10^4$ to $8.5 \times 10^4$ U/mg of protein. As the purities of the proteins were all >90%, as judged by SDS-polyacrylamide gel electrophoresis, the GyrB preparations seemed to contain a biologically inactive form. The reason why is not clear at present, but it may be related to the use of 6 M urea for solubilization of the GyrB protein or to a deficiency of chaperon proteins in the

overproducing strains. A similar phenomenon has been reported by Mizuuchi et al. (15).

The $ID_{50}$s of quinolones in supercoiling reactions were almost the same with pBR322 and pHY300PLK DNAs but were markedly different for the wild-type and mutant gyrases, suggesting that the quinolone sensitivities of the gyrases depended on gyrase subunits but not on substrate DNA. The quinolone sensitivities of the gyrases were well correlated with the quinolone susceptibilities of strains having the corresponding enzymes. It is interesting that the GyrA-type 2 GyrB double-mutant gyrase was more resistant to oxolinic acid than each single-mutant gyrase but was almost as sensitive to enoxacin and sparfloxacin as the wild-type gyrase. These results indicate that the quinolone sensitivities of the gyrases are determined by both GyrA and GyrB subunits in concert and depend on the chemical structures of the quinolones. The results support a previous hypothesis, in which the quinolone-gyrase interaction is assumed (29), more than they support the cooperative quinolone-DNA binding model, in which the interaction of a quinolone with single-stranded DNA and the self-association of quinolone molecules are assumed (21).

[$^3$H]Enoxacin binding studies with gel filtration revealed that enoxacin did not bind to gyrase alone or to DNA alone but did bind to the gyrase-DNA complexes. Such binding was first found by Shen et al. (20). Enoxacin binding to the gyrase-DNA complexes was saturable when the gyrase or DNA concentration was increased in the presence of fixed concentrations of the other components. In the experiment involving various enoxacin concentrations and fixed concentrations of DNA and gyrase, there seemed to be two phases of enoxacin binding to the wild-type gyrase-DNA and the type 2 GyrB mutant gyrase-DNA complexes but only one phase of enoxacin binding to the GyrA mutant gyrase-DNA and the type 1 GyrB mutant gyrase-DNA complexes. However, additional studies may be required to confirm this point because of the relatively small amounts of enoxacin bound. The total amounts of enoxacin bound to the former three complexes were similar, but those bound to the last complex were significantly lower than those bound to the former three complexes, results indicating that the amounts of enoxacin bound to gyrase-DNA complexes are not always correlated with the quinolone sensitivities of the gyrases. It is intriguing that enoxacin binding was detectable at concentrations as low as the MICs for strains having the corresponding gyrases.

A Scatchard plot analysis revealed that the enoxacin binding sites on the gyrase-DNA complexes seemingly did not exist until enoxacin was present. This result could be accounted for if the sites appeared because of conformational changes in the gyrase-DNA complexes during DNA supercoiling (20), so as to form a DNA-cleavable state, and quinolones stabilized the state, as suggested previously (1, 25). When we considered that there were two types of enoxacin binding sites, with high and low binding affinities, in the wild-type and the type 2 GyrB mutant gyrase-DNA complexes but that there was only one type of enoxacin binding site in the GyrA mutant gyrase-DNA complex and that all the $A^{KL16}$ molecules and 4% of the $B^{KL16}$ molecules consisted of gyrases active in enoxacin binding, as estimated from their specific activities, the high- and low-affinity binding sites in the former two complexes could be calculated to number about 1 and 3, respectively, per active gyrase and the binding site in the last complex could be calculated to number about 4 per active gyrase. This stoichiometry is not definite because of the difficulty in accurately determining the amounts of active gyrases and because enoxacin binding might not be at equilibrium throughout the gel filtration procedure used. Aside from such ambiguity, the amounts of enoxacin bound to gyrase-DNA complexes seemed to be as small as only a few molecules per active gyrase. This result supports the proposed idea that a subtle interaction between gyrases and quinolones would form a poison and might be sufficient to inhibit bacterial growth (4, 10, 24). The supercoiling inhibition assay for gyrases seems to be too insensitive to detect such a subtle quinolone-gyrase-DNA interaction.

An important finding was that the enoxacin binding affinities ($K_d$s) for the complexes, especially at low enoxacin concentrations, close to the MICs, were well correlated with the quinolone sensitivities of the gyrases and the MICs. The enoxacin binding affinities for gyrase-DNA complexes are probably determined by the interaction between quinolones and gyrase subunits. As enoxacin binding markedly decreased with the type 1 GyrB mutant gyrase, having a mutation of Asp-426 to Asn, and the enoxacin binding affinity for the type 2 GyrB mutant gyrase, having a mutation of Lys-447 to Glu, was higher than that for the wild-type gyrase, the piperazinyl group at position 7 of enoxacin is likely to be involved in an ionic interaction with the carboxyl group of Asp-426, as we proposed previously (29). This suggestion is also supported by the fact that enoxacin binding was markedly inhibited by sparfloxacin, which has a dimethylpiperazinyl group at position 7, but moderately inhibited by oxolinic acid, which lacks a basic group. This fact may explain why new quinolones, having a basic group at position 7, show more potent antibacterial and antigyrase activities than older quinolones, such as nalidixic acid and oxolinic acid, lacking a basic group. Quinolones appear to interact with the GyrA protein as well because a mutation, from Ser-83 to Leu, of the protein results in a decrease in the enoxacin binding affinity. Although the importance of the region near Ser-83 of GyrA has been suggested (28), the sites for interaction remain unidentified. It is well known that all active quinolones have 3-carboxyl and 4-oxo groups and form a chelation complex with divalent cations, such as $Mg^{2+}$ (18), which are necessary for the formation of gyrase-DNA complexes (5). As $Mg^{2+}$ ions would probably exist at the site at which DNA cleavage and rejoining occur, the ions might constitute another quinolone binding site.

From the results obtained, we propose a quinolone pocket model as the mechanism of action of quinolones, in which quinolones exert their action through their interaction in a pocket of the gyrase-DNA complex that appears during DNA cleavage-reunion reactions and quinolone binding affinities for the complex are determined by both GyrA and GyrB subunits in concert. Further studies are required to clarify the detailed structures of the quinolone binding sites in the pocket.

## REFERENCES

1. **Gellert, M.** 1981. DNA topoisomerases. Annu. Rev. Biochem. **50**:879–910.
2. **Gellert, M., L. M. Fisher, and M. H. O'Dea.** 1979. DNA gyrase: purification and catalytic activity of a fragment of gyrase B protein. Proc. Natl. Acad. Sci. USA **76**:6289–6293.
3. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Ito, and J. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74**:4772–4776.
4. **Hallett, P., and A. Maxwell.** 1991. Novel quinolone resistance mutations of the *Escherichia coli* DNA gyrase A protein: enzymatic analysis of the mutant proteins. Antimicrob. Agents

Chemother. **35**:335–340.
5. **Higgins, N. P., and N. R. Cozzarelli.** 1982. The binding of gyrase to DNA: analysis by retention by nitrocellulose filters. Nucleic Acids Res. **10**:6833–6847.
6. **Higgins, N. P., C. L. Peebles, A. Sugino, and N. R. Cozzarelli.** 1978. Purification of subunits of *Escherichia coli* DNA gyrase and reconstitution of enzymatic activity. Proc. Natl. Acad. Sci. USA **75**:1773–1777.
7. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
8. **Kamisky, D., and R. I. Meltzer.** 1968. Quinolone antibacterial agents. J. Med. Chem. **11**:160–163.
9. **Klevan, L., and J. C. Wang.** 1980. Deoxyribonucleic acid gyrase-deoxyribonucleic acid complex containing 140 base pairs of deoxyribonucleic acid and an $\alpha_2\beta_2$ protein core. Biochemistry **19**:5229–5234.
10. **Kreuzer, K. N., and N. R. Cozzarelli.** 1979. *Escherichia coli* mutants thermosensitive for deoxyribonucleic acid gyrase subunit A: effects on deoxyribonucleic acid replication, transcription, and bacteriophage growth. J. Bacteriol. **140**:424–435.
11. **Lesher, G. Y., E. J. Froelich, M. D. Gruett, J. H. Bailey, and R. P. Brundage.** 1962. 1,8-Naphthyridine derivatives. A new class of chemotherapeutic agents. J. Med. Pharm. Chem. **5**:1063–1065.
12. **Matsumoto, J., T. Miyamoto, A. Minamida, Y. Nishimura, H. Egawa, and H. Nishimura.** 1984. Pyridonecarboxylic acids as antibacterial agents. 2. Synthesis and structure-activity relationships of 1,6,7-trisubstituted 1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acids, including enoxacin, a new antibacterial agent. J. Med. Chem. **27**:292–301.
13. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
14. **Miyamoto, T., J. Matsumoto, K. Chiba, H. Egawa, K. Shibamori, A. Minamida, Y. Nishimura, H. Okada, M. Kataoka, M. Fujita, T. Hirose, and J. Nakano.** 1990. Synthesis and structure-activity relationships of 5-substituted 6,8-difluoroquinolones, including sparfloxacin, a new quinolone antibacterial agent with improved potency. J. Med. Chem. **33**:1645–1656.
15. **Mizuuchi, K., M. Mizuuchi, M. H. O'Dea, and M. Gellert.** 1984. Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. J. Biol. Chem. **259**:9199–9201.
16. **Mizuuchi, K., M. H. O'Dea, and M. Gellert.** 1978. DNA gyrase: subunit structure and ATPase activity of the purified enzyme. Proc. Natl. Acad. Sci. USA **75**:5960–5963.
17. **Nakamura, S., M. Nakamura, T. Kojima, and H. Yoshida.** 1989. *gyrA* and *gyrB* mutations in quinolone-resistant strains of *Escherichia coli*. Antimicrob. Agents Chemother. **33**:254–255.
18. **Okabayashi, Y., F. Hayashi, Y. Terui, and T. Kitagawa.** 1992. Studies on the interaction of pyridone carboxylic acids with metals. Chem. Pharm. Bull. **40**:692–696.
19. **Sato, K., Y. Inoue, T. Fujii, H. Aoyama, M. Inoue, and S. Mitsuhashi.** 1986. Purification and properties of DNA gyrase from a fluoroquinolone-resistant strain of *Escherichia coli*. Antimicrob. Agents Chemother. **30**:777–780.
20. **Shen, L. L., W. E. Kohlbrenner, D. Weigl, and J. Baranowski.** 1989. Mechanism of quinolone inhibition of DNA gyrase. J. Biol. Chem. **264**:2973–2978.
21. **Shen, L. L., L. A. Mitscher, P. N. Sharma, T. J. O'Donnell, D. W. T. Chu, C. S. Cooper, T. Rosen, and A. G. Pernet.** 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: a cooperative drug-DNA binding model. Biochemistry **28**:3886–3894.
22. **Shen, L. L., and A. G. Pernet.** 1985. Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. Proc. Natl. Acad. Sci. USA **82**:307–311.
23. **Sugino, A., C. L. Peebles, K. N. Kreuzer, and N. R. Cozzarelli.** 1977. Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. USA **74**:4767–4771.
24. **Walton, L., and L. P. Elwell.** 1988. In vitro cleavable-complex assay to monitor antimicrobial potency of quinolones. Antimicrob. Agents Chemother. **32**:1086–1089.
25. **Wang, J. C.** 1985. DNA topoisomerases. Annu. Rev. Biochem. **54**:665–697.
26. **Yamada, M., Y. Furutani, M. Notake, J. Yamagishi, M. Yamayoshi, T. Fukui, H. Nomura, M. Komiya, J. Kuwashima, K. Nakano, Y. Sohmura, and S. Nakamura.** 1985. Efficient production of human tumour necrosis factor in *Escherichia coli*. J. Biotechnol. **3**:141–153.
27. **Yamagishi, J., H. Yoshida, M. Yamayoshi, and S. Nakamura.** 1986. Nalidixic acid-resistant mutations of the *gyrB* gene of *Escherichia coli*. Mol. Gen. Genet. **204**:367–373.
28. **Yoshida, H., M. Bogaki, M. Nakamura, and S. Nakamura.** 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **34**:1271–1272.
29. **Yoshida, H., M. Bogaki, M. Nakamura, L. M. Yamanaka, and S. Nakamura.** 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **35**:1647–1650.
30. **Yoshida, H., T. Kojima, J. Yamagishi, and S. Nakamura.** 1988. Quinolone-resistant mutations of the *gyrA* gene of *Escherichia coli*. Mol. Gen. Genet. **211**:1–7.