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Mechanism of Inhibition of DNA Gyrase by Cyclothialidine, a Novel DNA Gyrase Inhibitor

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We investigated how cyclothialidine (Ro 09-1437), a novel DNA gyrase inhibitor belonging to a new chemical class of compounds, acts to inhibit *Escherichia coli* DNA gyrase. Cyclothialidine up to 100 μg/ml showed no effect on DNA gyrase when linear DNA was used as a substrate. Under the same conditions, quinolones, which inhibit the resealing reaction of DNA gyrase, caused a decrease in the amount of linear DNA used. No effect of cyclothialidine was observed on the accumulation of the covalent complex of DNA and the A subunit of DNA gyrase induced by ofloxacin in the absence of ATP. The effect of cyclothialidine on the DNA supercoiling reaction was antagonized by ATP, reducing the inhibitory activity 11-fold as the ATP concentration was increased from 0.5 to 5 mM. Cyclothialidine competitively inhibited the ATPase activity of DNA gyrase (K_i = 6 nM). The binding of [^{14}C]benzoyl-cyclothialidine to *E. coli* gyrase was inhibited by ATP and novobiocin, but not by ofloxacin. These results suggest that cyclothialidine acts by interfering with the ATPase activity of the B subunit of DNA gyrase. Cyclothialidine was active against a DNA gyrase resistant to novobiocin, suggesting that its precise site of action might be different from that of novobiocin.

DNA gyrase is a type II DNA topoisomerase that catalyzes the negative supercoiling of DNA in prokaryotes. Its function is essential to DNA replication, transcription, and bacteriophage λ integrative recombination (for reviews, see references 6, 14, and 27). A large body of evidence indicates that topoisomerases, including DNA gyrase, are the targets of therapeutically useful antibacterial and antitumor agents (5). The enzyme from *Escherichia coli* consists of two subunits, A and B, with molecular masses of 97,000 and 90,000 Da, respectively; the active enzyme is an A_2B_2 tetramer complex. Mechanistic studies have suggested that the following steps are involved in the DNA supercoiling process. The enzyme binds to DNA and forms a complex in which about 120 bp of DNA is wrapped around a protein core. Both strands of the wrapped DNA are then cleaved and covalent bonds are formed between the protein and the DNA (cleavable complex). A segment of DNA is passed through this break, and probably through part of the protein itself. Although gyrase requires the hydrolysis of ATP for the supercoiling of DNA, in its absence gyrase can relax negatively supercoiled DNA, albeit inefficiently. Replacement of ATP by the nonhydrolyzable ATP analog 5′-adenylyl-β-γ-imidodiphosphate (ADPNP) results in limited supercoiling by gyrase, suggesting that ATP binding can promote a single round of supercoiling, but the hydrolysis step is required to regenerate the enzyme in an active form (25).

DNA gyrase is the target of two classes of antibiotics: the synthetic quinolones, typified by nalidixic acid and the new fluoroquinolones, and the natural coumarins, such as novobiocin and coumermycin A1. Recently, three antibacterial agents, cinodine, microcin, and clerocidin, which fall outside the quinolone and coumarin classes have been reported to have DNA gyrase as their target (15, 18, 28). The quinolones are thought to act at the A subunit, probably by interfering with the DNA-rejoining step of the gyrase-mediated DNA strand-passing reaction (7, 20–22, 26). The coumarins are thought to act at the B subunit, probably by competing with ATP for binding to the B subunit of the enzyme (1, 3, 4, 8, 25).

From our screening of natural products for DNA gyrase inhibitors, we isolated a novel gyrase inhibitor, cyclothialidine, from *Streptomyces filipinensis* NR0484 (29). Cyclothialidine contains a unique 12-membered lactone ring that is partly integrated into a pentapeptide chain (see Fig. 4) (10). Cyclothialidine potently inhibited DNA gyrases from several bacterial species, including *E. coli* and *Staphylococcus aureus*, with a high degree of selectivity (17). In the study described here we investigated the mechanism by which cyclothialidine inhibits DNA gyrase and compared its mode of action with those of known DNA gyrase inhibitors, i.e., quinolones and coumarin antibiotics.

MATERIALS AND METHODS

Materials. Cyclothialidine was purified from a culture broth of *S. filipinensis* NR0484 and was shown to have >98% purity by high-pressure liquid chromatographic (HPLC) analysis (10). Novobiocin, coumermycin A1, distamycin A, ATP, ADPNP, and adenosine-5′-*O*-β-thiotriphosphate (ATPγS) were purchased from Sigma Chemical Company. Ofloxacin (Daiichi) and ciprofloxacin (Bayer) were >98% pure. The restriction endonuclease *Eco*RI was purchased from Takara Suzo Company.

DNA gyrase. Gyrase A and B subunits were purified separately from gyrase A and B subunit overproducing *E. coli* N4186 and MK47, respectively, by the method of Mizuuchi et al. (16). After valine-Sepharose chromatography, the gyrase A subunit-containing fraction contained minor amounts of gyrase B subunit. Therefore, it was applied to a novobiocin-Sepharose column as described by Staudenbauer and Orr (24), and the flowthrough fraction was collected. The gyrase B subunit was eluted from a hydroxylapatite column. Each sample was stored in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–KOH (pH 8.0) containing 1 mM dithiothreitol (DTT), 0.2 mM EDTA, and 50% (wt/vol) ethylene glycol at

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−70°C. The purified A subunit was judged to be >90% pure by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis; similarly, the purified B subunit was >95% pure. Gyrase activity was measured by a standard supercoiling assay (19). One unit was defined as the minimum amount of reconstituted gyrase that maximally supercoiled 0.5 μg of relaxed ColE1 DNA in 30 min at 30°C. The specific activities of the A and B subunits were 6.4×10^5 and 1.2×10^5 U/mg, respectively. Protein concentrations were determined by a Bio-Rad protein assay by using bovine serum albumin as a standard.

DNA gyrases for binding experiments and experiments with resistant DNA gyrases. DNA gyrases were partially purified from *E. coli* D110 (*thyA end*) (wild type), *E. coli* RK4429 [Δ(*tonB-trp*) Δ*lac*(*U119*) *araD metE non gyrA rpsL btuB btuC Zdh*::Tn5) (*gyrA*), and *E. coli* NBR2 (*gyrB*); *E. coli* NBR2 was spontaneously isolated from an *E. coli* D110 strain showing coumermycin A1 resistance in coumermycin A1-containing medium. Cells were lysed with lysozyme and Brij 58 as described by Otter and Cozzarelli (19), and the lysates were precipitated with ammonium sulfate. The 35 to 45% fraction was dialyzed against buffer A (25 mM HEPES–KOH [pH 8.0], 1 mM DTT, 1 mM EDTA, 10% [wt/vol] ethylene glycol, 0.5 mM phenylmethylsulfonyl fluoride, 0.15 M KCl) and fractionated by affinity column chromatography on novobiocin-Sepharose (24). One unit was defined as the minimum amount of gyrase that maximally supercoiled 0.5 μg of relaxed ColE1 DNA in 30 min at 30°C.

DNA. Supercoiled ColE1 DNA was purified from *E. coli* Hfr H5(ColE1/ColE1^r) by the alkaline lysis method (11) and was centrifuged in cesium chloride density gradients. Relaxed ColE1 DNA was conveniently prepared by treating this purified supercoiled DNA with the topoisomerase in a crude nuclear extract of rat liver in the presence of EDTA to inhibit nucleases (2, 19). Linear ColE1 DNA was prepared by cleavage with *Eco*RI according to the supplier's instructions. Bacteriophage λ DNA was purchased from Bethesda Research Laboratories and was then extracted with phenol and chloroform and precipitated with ethanol before use.

DNA rejoining assay. The DNA rejoining assay mixture (20 μl) contained 50 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 5 mM ATP, 5 mM spermidine · 3HCl, 50 μg of bovine serum albumin per ml, 17 fmol of *Eco*RI-restricted linear ColE1 DNA, and 5 U of reconstituted DNA gyrase. This reaction mixture was incubated for 30 min at 30°C, and then 2 μl of 5.5% SDS and 1 mg of proteinase K per ml were added to give final concentrations of 0.55% and 100 μg/ml, respectively. After incubation for an additional 30 min at 30°C, the reaction was stopped by adding 4 μl of a solution containing 37.5% (wt/vol) glycerol and 500 μg of bromophenol blue per ml. The reaction mixture was immediately loaded onto a 1% agarose gel and was electrophoresed. The amount of linear ColE1 DNA was quantitated by scanning the photographic negatives of the ethidium bromide-stained gels with a Shimadzu densitometer (model CS-920).

DNA-gyrase cleavable complex assay in the presence of ofloxacin. The drug-dependent effect was assayed by measuring the DNA-gyrase cleavable complex formed by gyrase in the presence of ofloxacin. The 20-μl reaction mixture contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 20 mM KCl, 2 mM DTT, 5 mM spermidine · 3HCl, 50 μg of bovine serum albumin per ml, 125 μg of *E. coli* tRNA per ml, 0.5 μg of relaxed ColE1 DNA, 2 U of reconstituted DNA gyrase, and 100 μg of ofloxacin per ml. The reaction mixture was incubated for 30 min at 30°C and was then loaded onto a 0.8% agarose gel and electrophoresed.

DNA-supercoiling assay. The DNA-supercoiling assay for DNA gyrase was performed as described elsewhere (17). The formation of supercoiled ColE1 DNA bands was analyzed by using 0.8% agarose gels.

ATPase assay. [γ-^{32}P]ATP (specific activity, 6,000 Ci/mmol) and [^{3}H]glycine (specific activity, 18 Ci/mmol) were purchased from Amersham. [γ-^{32}P]ATPase was assayed by following the method of Maxwell and Gellert (13). The ATPase reaction mixtures contained 20 or 60 nM B subunit, a twofold molar excess of the A subunit, 10 or 30 μg of linear bacteriophage λ DNA per ml, 0.05 to 25 mM ATP, [γ-^{32}P]ATP (100 nCi), and 0.06 to 0.12 μM [^{3}H]glycine (30 to 60 nCi) to normalize the data and to compensate for pipetting errors in a buffer containing 35 mM Tris-HCl (pH 7.6), 24 mM KCl, 6 mM $MgCl_2$, 1.8 mM spermidine, 0.27 mg of bovine serum albumin per ml, 9 μg of *E. coli* tRNA per ml, 6.5% (wt/vol) ethylene glycol, 5 mM DTT, and 10 mM potassium phosphate. Reaction mixtures containing DNA gyrase were incubated for 30 min at 25°C prior to the addition of ATP and DNA. Samples of 30 μl were withdrawn at various times and placed in 1 ml of an ice-cold 16% (wt/vol) suspension of activated charcoal in water, and the mixtures were left on ice with occasional shaking for 10 min. Samples were then spun in a microcentrifuge for 10 min, 0.5 ml of the supernatant was withdrawn, and the amounts of labelled phosphate and glycine were determined by scintillation counting. Less than 5% glycine and more than 99.5% ATP bound to the activated charcoal. These control experiments were done under the same conditions as the assay but without cold ATP, DNA, and DNA gyrase. The binding of P_i was prevented by the addition of 10 mM potassium phosphate to the reaction buffer, and the measured release of P_i was always less than 0.1 mM. Results were calculated as the ratio of ^{32}P/^{3}H counts and were converted to the percentage of ATP hydrolyzed by comparison with the ^{32}P/^{3}H ratio of an untreated reaction. Initial rates were determined from time points taken in the early part of the reaction (<5% hydrolysis).

[^{14}C]benzoyl-cyclothialidine. [^{14}C]benzoyl-cyclothialidine was prepared by the reaction of cyclothialidine with [^{14}C]benzoic acid (21.8 mCi/mmol) in the presence of *N,N'*-disuccinimidyl carbonate in acetonitrile-pyridine (1:1) and was found to have >98% purity (see Fig. 4). The labelled compound had a specific activity of 21.8 mCi/mmol. Its mobilities on thin-layer chromatography (TLC) and HPLC were identical to those of authentic benzoyl-cyclothialidine. Benzoyl-cyclothialidine has almost the same inhibitory activity as cyclothialidine against *E. coli* DNA gyrase (data not shown).

[^{14}C]benzoyl-cyclothialidine-binding experiment. The binding of [^{14}C]benzoyl-cyclothialidine was determined by a centrifugal filtration method (21, 23). Centrifree micropartition devices (Amicon no. 4103) were used to separate [^{14}C]benzoyl-cyclothialidine bound to DNA gyrase from the free ligand. Reactions (reaction mixture, 400 μl) were carried out as described above for the supercoiling assay, but without ATP, and contained an appropriate amount of DNA gyrase and radioactive ligand in standard buffer (50 mM Tris-HCl [pH 8.0], 20 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT). After incubation for 30 min at 30°C, the mixtures were transferred to the Centrifree devices and were centrifuged at 1,600 × *g* in a Kubota KR-180B (swinging bucket rotor) for 30 min at 4°C. The membrane disks and O rings of the Centrifree devices were placed in vials with 1 ml of standard buffer. The vials were shaken on a rotary shaker for 2 h to solubilize the [^{14}C]benzoyl-cyclothialidine. Then, 15 ml of liquid scintillator {toluene-ethanol [1:1], 0.7% butyl-PBD [2-(4-tert-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole]} was added to each

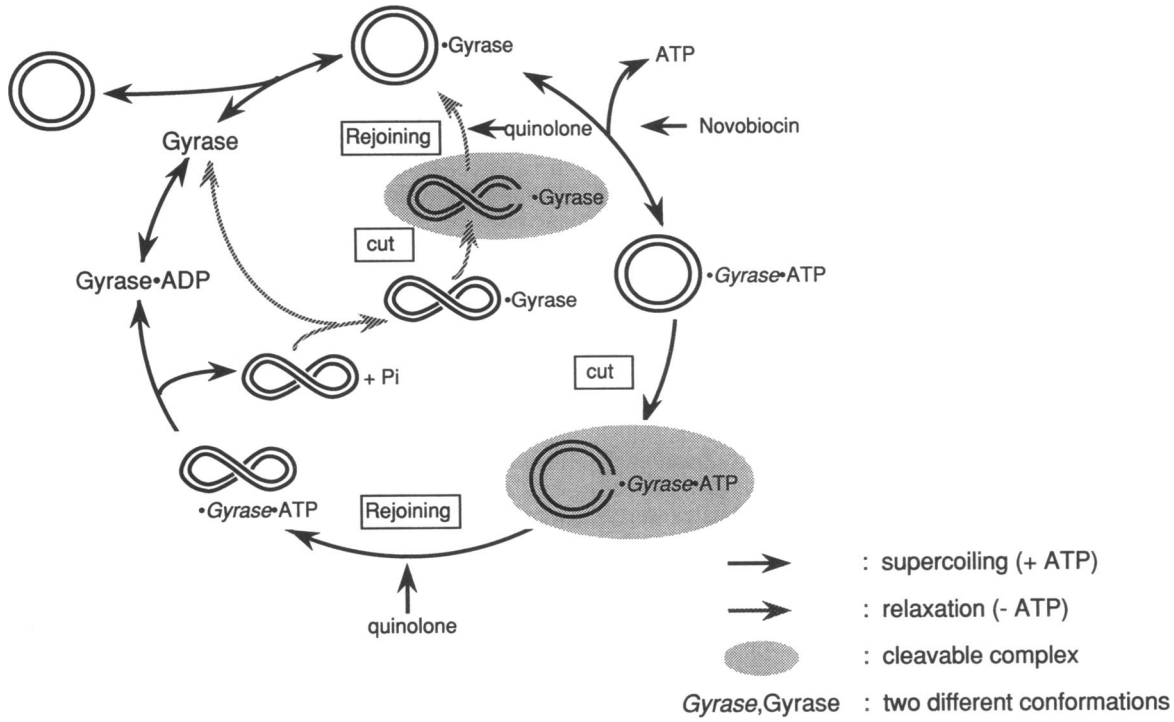


FIG. 1. DNA gyrase reactions and sites of action of gyrase inhibitors.

vial to measure the radioactivity. To determine the nonspecific ligand binding, the assay was run with excess cyclothialidine. The amount of bound ligand was calculated after subtracting the nonspecific bound radioactivity.

RESULTS

Cyclothialidine does not inhibit the rejoining step of DNA. We investigated whether cyclothialidine acts similarly to the quinolone drugs (Fig. 1). It is well established that quinolone drugs such as ofloxacin inhibit the DNA-rejoining step of the gyrase-mediated DNA strand-passing reaction (Fig. 1). The A subunit is likely to be the site of action of quinolones because it carries out the DNA breakage and rejoining steps. When gyrase and DNA are incubated together in the presence of ofloxacin, the so-called gyrase-DNA cleavable complex is formed, which, after the addition of SDS, releases DNA that is cleaved in both strands (19). To detect the release of cleaved DNA from this cleavable complex easily, we used linear ColE1 DNA in place of relaxed ColE1 DNA and then measured the decrease in the amount of this linear ColE1 DNA caused by the drug. Ofloxacin at a concentration of 0.6 μg/ml resulted in a 50% decrease in the amount of the linear DNA used, whereas cyclothialidine, similar to novobiocin and coumermycin A1 at concentrations up to 100 μg/ml caused no decrease in the amount of linear DNA, indicating that under these conditions, in which ofloxacin stabilizes the gyrase-DNA cleavable complex, cyclothialidine does not appear to do so. This result was quite consistent with our previous findings (17) that cyclothialidine does not produce the gyrase-DNA cleavable complex like quinolones do in the DNA supercoiling assay. Therefore, we concluded that the mode of action of cyclothialidine as well as its structure are different from those of the quinolones.

Cyclothialidine does not inhibit the DNA-binding or the DNA-cutting step of DNA gyrase. We used the cleavable complex assay to examine whether cyclothialidine inhibits the DNA-cutting step or even DNA binding (Fig. 1). As described above, quinolones such as ofloxacin arrest DNA gyrase at the cleavage step, resulting in the accumulation of a ternary complex of DNA, gyrase, and quinolone. If cyclothialidine were to inhibit one of the previous steps, the amount of cleavable complex formed in the presence of both cyclothialidine and ofloxacin should be decreased. To simplify the reaction, ATP was depleted from the assay mixture, and distamycin A, a DNA-binding agent, was used as a reference compound. Distamycin A inhibits gyrase in the DNA-supercoiling reaction, with 50% inhibitory concentration (IC_{50}) of 3 μg/ml (data not shown). In the DNA cleavable complex assay carried out in the presence of 100 μg of ofloxacin per ml, distamycin A prevented the accumulation of cleavable complexes. The formation of cleavable complexes was fully inhibited at a concentration of 10 μg of distamycin A per ml (Fig. 2). It is predicted that distamycin A probably inhibits the DNA gyrase reaction at one of the initial steps, such as by preventing DNA gyrase from binding to DNA or from moving to the DNA-cutting sites. In contrast, the amount of cleavable complex remained constant in the presence of another reference compound, novobiocin, which is known to inhibit the ATPase activity of gyrase, even when the concentration of novobiocin was increased to 10 μg/ml (IC_{50} in the DNA-supercoiling reaction, 0.06 μg/ml). Cyclothialidine in the range from 0.2 to 10 μg/ml also showed no effect on the accumulation of the cleavable complex (IC_{50} in the DNA-supercoiling reaction, 0.03 μg/ml) (17). Thus, we conclude that cyclothialidine inhibits neither the DNA-binding nor the DNA-cutting step of DNA gyrase.

Antagonism with ATP in the DNA-supercoiling reaction. DNA gyrase requires ATP for the DNA-supercoiling reaction, and it is well established that the B subunit catalyzes the

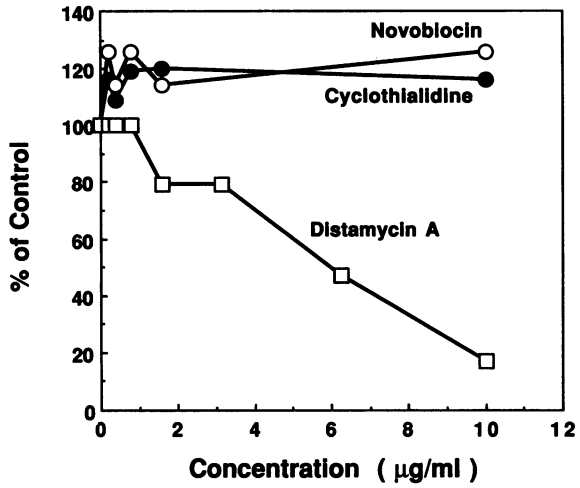


FIG. 2. Effects of inhibitors on the formation of DNA-gyrase cleavable complex in the presence of ofloxacin. Reactions were as described in Materials and Methods, except for the addition of the indicated amounts of cyclothialidine (●), novobiocin (○), or distamycin A (□). The accumulation of the DNA-gyrase-cleavable complex was quantitated as described in Materials and Methods for the DNA-rejoining assay.

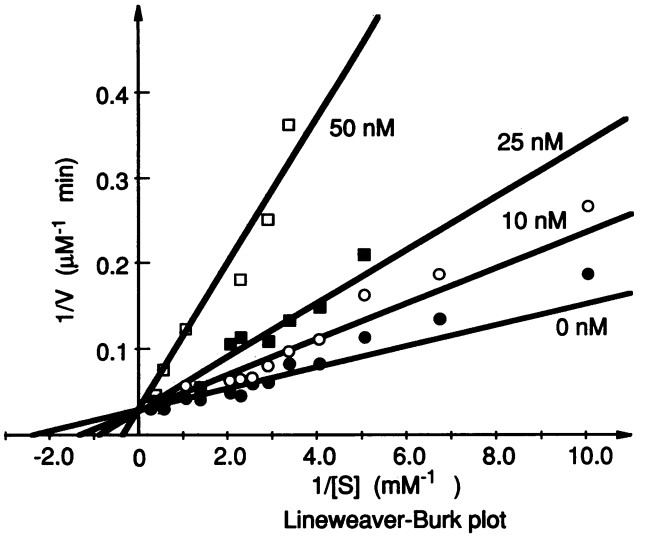


FIG. 3. Competitive inhibition of the ATPase activity of *E. coli* DNA gyrase by cyclothialidine. Reactions were as described in Materials and Methods. The ATPase reaction mixtures contained the indicated amounts of ATP and no cyclothialidine (●), 10 nM cyclothialidine (○), 25 nM cyclothialidine (■), or 50 nM cyclothialidine (□). After incubation at 25°C, samples were withdrawn at various times and the release of $^{32}P_i$ was measured by scintillation counting.

hydrolysis of ATP (19). Therefore, it is possible that cyclothialidine inhibits the ATPase activity of gyrase. We did a preliminary test to see whether cyclothialidine inhibition of the DNA-supercoiling reaction was antagonized by ATP. When the ATP concentration was increased from 0.5 to 5 mM, the IC_{50}s of the B-subunit inhibitor novobiocin increased 13-fold (from an IC_{50} of 0.09 μg/ml in 0.5 mM ATP to an IC_{50} of 1.2 μg/ml in 5 mM ATP). In contrast, with an A-subunit inhibitor, ciprofloxacin, the IC_{50} was not affected so much, increasing only 2.8-fold (from an IC_{50} of 0.40 μg/ml in 0.5 mM ATP to an IC_{50} of 1.1 μg/ml in 5 mM ATP). The inhibitory activity of cyclothialidine was also antagonized by ATP, showing an 11-fold increase in the IC_{50}, from 0.048 μg/ml in 0.5 mM ATP to 0.55 μg/ml in 5 mM ATP, which is similar to the result obtained with novobiocin (Table 1).

Cyclothialidine is competitive with ATP. To study further the ATP antagonism for cyclothialidine inhibition shown in Table 1, we measured the ATPase activity of DNA gyrase. We confirmed the results of Maxwell and Gellert (13), that the B subunit alone has a very low level of ATPase activity which is highly stimulated by the addition of the A subunit and DNA. In our case we measured at least a 20-fold increase in the ATPase activity (data not shown). If cyclothialidine simply prevents ATP binding, it should be a competitive inhibitor, similar to novobiocin and coumermycin A1. To test this hypothesis, we measured the ATPase activity in the presence of different concentrations of cyclothialidine. The intersection on the ordinate of the family of lines in the Lineweaver-Burk plot in Fig. 3 indicates that under steady-state conditions cyclothialidine indeed seems to compete with ATP. The K_i value for cyclothialidine derived from these ATPase data is 6×10^{-9} M. This value is comparable to the K_i values for the B-subunit inhibitors novobiocin (1×10^{-8} M) and coumermycin A1 (4.5×10^{-9} M) (25). Therefore, cyclothialidine is a B-subunit inhibitor, similar to novobiocin and coumermycin A1. The efficacies of cyclothialidine, novobiocin, and coumermycin A1 as inhibitors of the ATPase activity closely correlated with their potencies in the DNA-supercoiling assay (17), implying that the inhibition of DNA supercoiling, which includes the whole DNA gyrase reaction process, resulted from the inhibition of the ATPase by the B-subunit inhibitors.

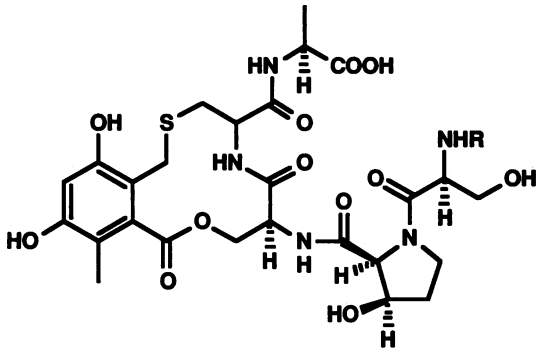
TABLE 1. Effect of ATP on DNA-supercoiling inhibitory activity[a]

Compound	IC_{50} (μg/ml)		Ratio of IC_{50} in 5 mM ATP/IC_{50} in 0.5 mM ATP
	0.5 mM ATP	5 mM ATP	
Cyclothialidine	0.048	0.55	11.5
Novobiocin	0.090	1.20	13.3
Ciprofloxacin	0.40	1.10	2.8

[a] DNA-supercoiling reactions were as described in Materials and Methods, except that 0.5 μg of relaxed ColE1 DNA, 4 U of reconstituted DNA gyrase, 0.5 or 5 mM ATP, and several concentrations of cyclothialidine, novobiocin, and ciprofloxacin were used. The IC_{50} is defined as the inhibitory concentration at which the intensity of the supercoiled DNA band was half that of the control.

[^{14}C]benzoyl-cyclothialidine binding to gyrase. To further characterize the mechanism of cyclothialidine inhibition, we examined the binding of [^{14}C]benzoyl-cyclothialidine to *E. coli* DNA gyrase. The structures of [^{14}C]benzoyl-cyclothialidine and cyclothialidine are shown in Fig. 4. The [^{14}C]benzoyl-cyclothialidine binding increased in proportion to the amount of DNA gyrase added (data not shown). When a 250-fold excess of unlabelled cyclothialidine was added, the amount of [^{14}C]benzoyl-cyclothialidine bound to DNA gyrase was decreased to a level at which it could hardly be detected (Fig. 5), indicating that [^{14}C]benzoyl-cyclothialidine binds reversibly to DNA gyrase. This suggests that cyclothialidine also binds reversibly to the DNA gyrase. However, the half-life of the dissociation could not be exactly determined in this experiment, because the membrane filtration step by centrifugation took 30 min, although most of the reaction mixture passed through the membrane in 10 min.

Then, we studied the effects of cyclothialidine, novobiocin,



Compound	R
Cyclothialidine	H
^{14}C-benzoyl-cyclothialidine	7-^{14}C-benzoyl

FIG. 4. Structures of cyclothialidine and [^{14}C]benzoyl-cyclothialidine.

ATPγS, and ofloxacin on [^{14}C]benzoyl-cyclothialidine binding. Binding was inhibited by cyclothialidine, novobiocin, and ATPγS, with IC_{50}s of 1.1×10^{-7}, 5.9×10^{-7}, and 2.2×10^{-3} M, respectively, whereas ofloxacin did not inhibit binding at concentrations of up to 1×10^{-1} M (Fig. 6A and B). From the Scatchard analysis, [^{14}C]benzoyl-cyclothialidine was found to bind to DNA gyrase with a K_d value of 2.8×10^{-8} M, and the binding capacity (B_{max}) of approximately 1 pmol/pmol of the B subunit indicated that [^{14}C]benzoyl-cyclothialidine binds to the B subunit in a one-to-one stoichiometric fashion (Fig. 7). A similar K_d (1.8×10^{-9} M) and one-to-one stoichiometry were also obtained with a highly purified enzyme. Moreover, in the presence of novobiocin, the B_{max} of [^{14}C]benzoyl-cyclothialidine was invariant (17a), although the decreasing slope of the Scatchard plot was dependent on the concentration of novobiocin.

Inhibitory activity against DNA gyrases isolated from resistant *gyrA* and *gyrB* mutants of *E. coli*. To learn whether the inhibition mechanism of cyclothialidine is the same as those of other known gyrase inhibitors, we tested the inhibitory activity of cyclothialidine against gyrases isolated from *E. coli* strains

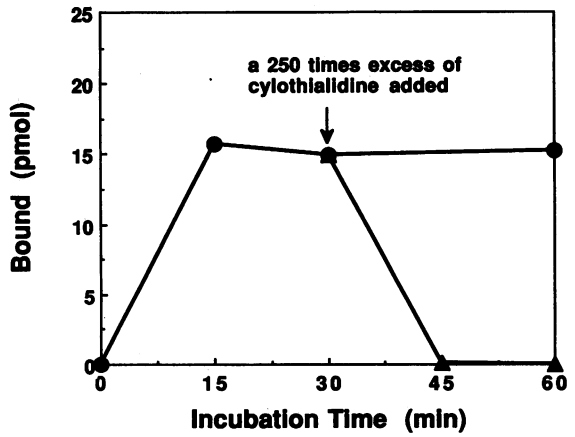


FIG. 5. Association and dissociation of [^{14}C]benzoyl-cyclothialidine binding to *E. coli* DNA gyrase. Experiments were performed as described in Materials and Methods. Reaction mixtures contained 8.3 pmol of the A subunit, 25.5 pmol of the B subunit, and 1.3×10^{-7} M [^{14}C]benzoyl-cyclothialidine. The time course for [^{14}C]benzoyl-cyclothialidine binding to DNA gyrase was examined for up to 60 min. After 30 min of incubation, one aliquot was withdrawn and a 250 times excess of unlabelled cyclothialidine (1 mg/ml, 10 μl) was added to the reaction mixture, which was incubated for an additional 30 min.

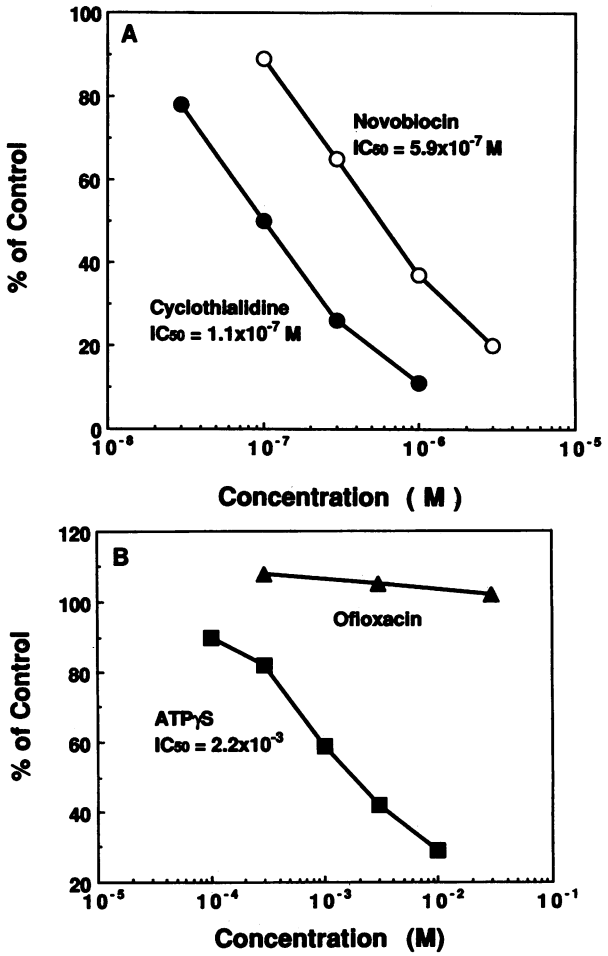


FIG. 6. Effects of inhibitors on [^{14}C]benzoyl-cyclothialidine binding to *E. coli* DNA gyrase. Experiments were performed as described in Materials and Methods. Reaction mixtures contained 8.3 pmol of the A subunit, 25.5 pmol of the B subunit, 1.3×10^{-7} M [^{14}C]benzoyl-cyclothialidine, and the indicated amounts of cyclothialidine (●) and novobiocin (○) (A) or ATPγS (■) and ofloxacin (▲) (B). After 30 min at 30°C, the levels of bound [^{14}C]benzoyl-cyclothialidine were determined by the centrifugal filtration method.

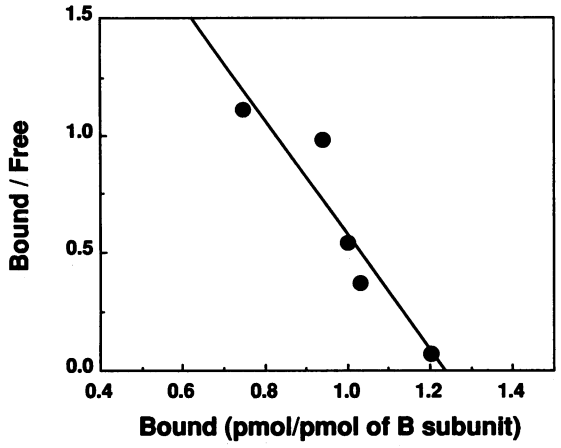


FIG. 7. Scatchard analysis of [^{14}C]benzoyl-cyclothialidine binding to *E. coli* DNA gyrase. Reactions were as described in the legend to Fig. 6 and Materials and Methods, except that 11 pmol of the A subunit, 34 pmol of the B subunit, and 0.075 to 1 μg of [^{14}C]benzoyl-cyclothialidine per ml were added.

resistant to either quinolone (*gyrA*) or novobiocin (*gyrB*). The gyrase from the *gyrA* mutant did not show resistance to cyclothialidine, whereas it was 10-fold more resistant than the wild-type gyrase to ofloxacin. Gyrase from the *gyrB* mutant was inhibited by cyclothialidine as potently as the gyrase from the parent strain was. However, the *gyrB* gyrase was 16-fold more resistant than the wild-type gyrase to novobiocin and coumermycin A1 (Table 2). Because a mutation in the *gyrB* gene was confirmed by the resistance of *gyrB* gyrase to the B-subunit inhibitors novobiocin and coumermycin A1 (17a), the precise action site of cyclothialidine seems to be different from those of the coumarin-type antibiotics.

DISCUSSION

Characterization of the mode of interaction of DNA gyrase with the novel inhibitor cyclothialidine promises to yield important information on the mechanism of DNA supercoiling.

DNA replication directed from the *oriC* origin of *E. coli* was inhibited by cyclothialidine (17). However, the activity of cyclothialidine in the DNA replication assay was weaker than its inhibitory activity against DNA gyrase (IC_{50} replication/ IC_{50} gyrase = 11.7). Because similar activity ratios were observed with the B-subunit inhibitors novobiocin and coumermycin A1 but not with the A-subunit inhibitor ofloxacin, it appeared that cyclothialidine might have a mode of action similar to those of the coumarin-type drugs in attacking the B subunit. The similarity of the mode of action of cyclothialidine to the known B-subunit inhibitors was further reinforced by the following four results. First, cyclothialidine did not interfere with the DNA-rejoining reaction of DNA gyrase that is inhibited by quinolones. Second, neither cyclothialidine nor novobiocin affected the accumulation of DNA-gyrase complexes produced by the action of ofloxacin (Fig. 2). Third, ATP reversed the inhibitory activity of cyclothialidine and novobiocin in the DNA-supercoiling assay (Table 1). Finally, the results of the ATPase assay with DNA gyrase indicate that cyclothialidine may compete with ATP. Thus, it was shown that cyclothialidine is a B-subunit inhibitor whose inhibitory mechanism is quite similar to those of the coumarin-type antibiotics. However, two important differences were observed between cyclothialidine and the coumarin-type antibiotics. First, cyclothialidine was much more selective than novobiocin and coumermycin A1 for the DNA gyrase (17). Cyclothialidine inhibited *E. coli* DNA gyrase potently, with an IC_{50} of 0.03 μg/ml; furthermore, cyclothialidine showed high selectivity toward DNA gyrase in comparison with its inhibition of DNA topoisomerases I and II of calf thymus, with IC_{50}s of 1,700 and 1,900 μg/ml, respectively. However, novobiocin inhibited those human topoisomerases with IC_{50}s of 760 and 400 μg/ml, respectively, and coumermycin A1 inhibited them with IC_{50}s of 39 and 75 μg/ml, respectively. This highly selective activity of cyclothialidine was confirmed by the evaluation with two nucleic acid polymerases, *E. coli* RNA polymerase and HeLa DNA polymerase α. Cyclothialidine did not inhibit these two enzymes (IC_{50}s, greater than 1,000 μg/ml), while novobiocin inhibited these enzymes with IC_{50}s of 300 to 400 μg/ml and coumermycin A1 inhibited them with IC_{50}s of 20 to 40 μg/ml. Second, despite its resemblance to novobiocin in its mechanism of action, cyclothialidine was active against the DNA gyrase isolated from a novobiocin-resistant *E. coli gyrB* mutant (Table 2). These facts show that cyclothialidine is novel not only in its structure but also in its mode of action.

[^{14}C]benzoyl-cyclothialidine was shown to bind to DNA gyrase in direct proportion to the gyrase concentration in the absence of DNA. This was the first direct evidence that cyclothialidine binds to the gyrase and that this binding site could be distinguished from the nonspecific cyclothialidine-binding sites on proteins and membranes. The association-dissociation profile of [^{14}C]benzoyl-cyclothialidine binding showed that this binding is reversible (Fig. 5). Also, the results

TABLE 2. Inhibitory activity against DNA gyrases from wild-type and resistant *E. coli* strains[a]

Compound	IC_{100} (μg/ml) for wild-type gyrase	Relative IC_{100} for gyrases from:		
		Susceptible (wild-type) strain	Nalidixic acid-resistant strain (*gyrA*)	Novobiocin resistant strain (*gyrB*)
Cyclothialidine	3.13	1	1	1
A-subunit inhibitor, ofloxacin	50	1	10[b]	1
B-subunit inhibitors				
Novobiocin	3.13	1	1	16
Coumermycin A1	3.13	1	1	16

[a] DNA-supercoiling reactions were as described in Materials and Methods, except that 0.5 μg of relaxed ColE1 DNA, 1 U of DNA gyrase prepared from each *E. coli* strain, 5 mM ATP, and several concentrations of cyclothialidine, ofloxacin, novobiocin, and coumermycin A1 were used. The IC_{100} is defined as the minimum inhibitory concentration at which a supercoiled band of DNA completely disappeared from the agarose gel. The relative IC_{100} expresses the relative ratio of the IC_{100} for resistant gyrase to the IC_{100} for wild-type gyrase when the IC_{100} for wild-type gyrase is set to 1.

[b] Ratio of IC_{50}s.

of the binding experiment strongly suggested that cyclothialidine inhibits the binding of ATP to the enzyme and acts via the B subunit. However, it is not yet known whether the binding of [^{14}C]benzoyl-cyclothialidine requires both subunits of the gyrase or only the B subunit, nor is it known whether it has an effect on DNA. We are now investigating these aspects by using a system of reconstituted *E. coli* gyrase subunits plus DNA. The K_d value of 2.8×10^{-8} M for the binding of [^{14}C]benzoyl-cyclothialidine to the cyclothialidine-binding site on gyrase is in good accord with the IC_{50} of 0.03 μg/ml (4.6×10^{-8} M) for the DNA-supercoiling activity of gyrase.

However, there are some differences between the K_d value of 2.8×10^{-8} M for [^{14}C]benzoyl-cyclothialidine and the K_i value of 6×10^{-9} M for cyclothialidine derived from the ATPase data. The inhibition of the DNA-supercoiling activity of gyrase by benzoyl-cyclothialidine is almost the same as that by cyclothialidine. For the ATPase of *E. coli* DNA gyrase, it is known that the ATPase activity is observed only in the presence of DNA (13), indicating a change in affinity for ATP by DNA. The differences between K_d and K_i might be resolved if we did the binding experiments with a system of reconstituted gyrase plus DNA and clarified what the effects of the A subunit and DNA on the binding are. It might be possible that the affinity for cyclothialidine is also affected by the molecules that constitute DNA gyrase activity.

Wigley et al. (30) reported that a 43-kDa N-terminal fragment of the B subunit of the *E. coli* DNA gyrase comprising positions 2 to 293 of the intact protein has been crystallized in the presence of ADPNP. With the observed interactions between ADPNP and the protein in the crystal structure, a lot of structural information about the ATP-binding site has been obtained, and these interactions also suggest that protein dimerization is required to bind ATP. These observations have important implications for the mechanism of supercoiling by gyrase. del Castillo et al. (4) have previously reported that three independent substitutions conferring resistance to coumermycin affect the arginine residue at position 136 (Arg-136) (mutations to Leu or Cys). Contreras and Maxwell (3) have also reported that Arg-136 and Gly-164, the two residues shown to be involved in coumarin resistance in *E. coli*, lie outside the ATP-binding site of the B subunit. Furthermore, recent results of Ali et al. (1) on steady-state kinetic experiments, in which the 43-kDa N-terminal fragment of the B subunit was used, are consistent with the idea of a noncompetitive mechanism for the inhibition of the ATPase activity of the B subunit by the coumarin drugs. This indicates that coumarin drugs bind closely to the ATPase site of the DNA gyrase B subunit and stabilize a conformation of the protein with a low affinity for ATP (12). From our results, we suggest that cyclothialidine and the coumarins bind to the B subunit and stabilize a conformation that is unable to bind nucleoside triphosphate and also suggest that cyclothialidine recognizes a site on the B subunit which is different from that recognized by the coumarins. An understanding of the molecular details of these interactions, however, awaits further studies.

All of the results obtained so far indicate that cyclothialidine is a promising lead compound for chemical modification, with the aim of creating a new class of antibacterial agents. Chemical programs for identifying the minimum structural requirements for activity and for enhancing in vivo penetrability into cells are being pursued (9).

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REFERENCES

1. **Ali, J. A., A. P. Jackson, A. J. Howells, and A. Maxwell.** 1993. The 43-kilodalton N-terminal fragment of the DNA gyrase B protein hydrolyzes ATP and binds coumarin drugs. Biochemistry **32:**2717–2724.
2. **Blobel, G., and U. Potter.** 1966. Nuclei from rat liver: isolation method that combines purity with high yield. Science **154:**1662–1665.
3. **Contreras, A., and A. Maxwell.** 1992. Gyr B mutations which confer coumarin resistance also affect DNA supercoiling and ATP hydrolysis by *Escherichia coli* DNA gyrase. Mol. Microbiol. **6:**1617–1624.
4. **del Castillo, I., J. L. Vizán, M. C. Rodríguez-Sáinz, and F. Moreno.** 1991. An unusual mechanism for resistance to the antibiotic coumermycin A1. Proc. Natl. Acad. Sci. USA **88:**8860–8864.
5. **Drlica, K., and R. J. Franco.** 1988. Inhibitors of DNA topoisomerases. Biochemistry **27:**2253–2259.
6. **Gellert, M.** 1981. DNA topoisomerases. Annu. Rev. Biochem. **50:**879–910.
7. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74:**4772–4776.
8. **Gellert, M., M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA **73:**4474–4478.
9. **Götschi, E., P. Angehrn, H. Gmünder, P. Hebeisen, H. Link, R. Masciadri, and J. Nielsen.** 1993. Cyclothialidine and its congeners: a new class of DNA gyrase inhibitors. Pharmacol. Ther. **60:**367–380.
10. **Kamiyama, T., N. Shimma, T. Ohtsuka, N. Nakayama, Y. Itezono, N. Nakada, J. Watanabe, and K. Yokose.** 1994. Cyclothialidine, a novel DNA gyrase inhibitor. II. Isolation, characterization and structure elucidation. J. Antibiot. **47:**37–45.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
12. **Maxwell, A.** 1993. The interaction between coumarin drugs and DNA gyrase. Mol. Microbiol. **9:**681–686.
13. **Maxwell, A., and M. Gellert.** 1984. The DNA dependence of the ATPase activity of DNA gyrase. J. Biol. Chem. **259:**14472–14480.
14. **Maxwell, A., and M. Gellert.** 1986. Mechanistic aspects of DNA topoisomerases. Adv. Protein Chem. **38:**69–107.
15. **McCullough, J. E., M. T. Muller, A. J. Howells, A. Maxwell, J. O'Sullivan, R. S. Summerill, W. L. Parker, J. S. Wells, D. P. Bonner, and P. B. Fernandes.** 1993. Clerocidine, a terpenoid antibiotic, inhibits bacterial DNA gyrase. J. Antibiot. **46:**526–530.
16. **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.
17. **Nakada, N., H. Shimada, T. Hirata, Y. Aoki, T. Kamiyama, J. Watanabe, and M. Arisawa.** 1993. Biological characterization of cyclothialidine, a new DNA gyrase inhibitor. Antimicrob. Agents Chemother. **37:**2656–2661.

17a. **Nakada, N., et al.** Unpublished data.

18. **Osburne, M. S., W. M. Maiese, and M. Greenstein.** 1990. In vitro inhibition of bacterial DNA gyrase by cinodine, a glycosinnamosylspermidine antibiotic. Antimicrob. Agents Chemother. **34:**1450–1452.
19. **Otter, R., and N. R. Cozzarelli.** 1983. *Escherichia coli* DNA gyrase. Methods Enzymol. **100:**171–180.
20. **Shen, L. L., J. Baranowski, and A. G. Pernet.** 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: specificity and cooperativity of drug binding to DNA gyrase. Biochemistry **28:**3879–3885.
21. **Shen, L. L., W. E. Kohlbrenner, D. Weigh, and J. Baranowski.** 1989. Mechanism of quinolone inhibition of DNA gyrase. J. Biol. Chem. **264:**2973–2978.
22. **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.

23. **Shen, L. L., and A. G. Pernet.** 1985. Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drug is DNA. Proc. Natl. Acad. Sci. USA **82**:307–311.
24. **Staudenbauer, W. L., and E. Orr.** 1981. DNA gyrase: affinity chromatography on novobiocin-Sepharose and catalytic properties. Nucleic Acids Res. **9**:3589–3603.
25. **Sugino, A., N. Higgins, P. Brown, C. Peebles, and N. R. Cozzarelli.** 1978. Energy coupling in DNA gyrase and the mechanism of action of novobiocin. Proc. Natl. Acad. Sci. USA **75**:4838–4842.
26. **Sugino, A., C. Peebles, K. Kreuzer, and N. R. Cozzarelli.** 1977. Mechanism of action of nalidixic acid: purification of *Escherichia coli* nal A gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. USA **74**:4767–4771.
27. **Sutcliffe, J. A., T. D. Gootz, and J. F. Barrett.** 1989. Biochemical characteristic and physiological significance of major DNA topoisomerase. Antimicrob. Agents Chemother. **33**:2027–2033.
28. **Vizán, J. L., C. Hernández-Chico, I. del Castillo, and F. Moreno.** 1991. The peptide antibiotic microcin B17 induces double-strand cleavage of DNA mediated by *E. coli* DNA gyrase. EMBO J. **10**:467–476.
29. **Watanabe, J., N. Nakada, S. Sawairi, H. Shimada, S. Ohshima, T. Kamiyama, and M. Arisawa.** 1994. Cyclothialidine, a novel DNA gyrase inhibitor. I. Screening, taxonomy, fermentation and biological activity. J. Antibiot. **47**:32–36.
30. **Wigley, D. B., G. J. Davies, E. J. Dodson, A. Maxwell, and G. Dodson.** 1991. Crystal structure of an N-terminal fragment of the DNA gyrase B protein. Nature (London) **35**:624–629.