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Differential Effects of Antibiotics Inhibiting Gyrase

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Both oxolinic acid and coumermycin A_1, inhibitors of DNA gyrase, block DNA synthesis in *Escherichia coli*. At low concentrations of oxolinic acid, the rate of bacterial DNA synthesis first declines rapidly but then gradually increases. This gradual increase in synthesis rate depended on the presence of wild-type *recA* and *lexA* genes; mutations in either gene blocked the increase in synthesis rate. In such mutants, oxolinic acid caused a rapid decline, followed by a slow, further decrease in DNA synthesis rate. Coumermycin A_1, however, produced a more gradual decline in synthesis rate which is unaffected by defects in the *recA* or *lexA* genes. An additional difference between the two drugs was observed in a *dnaA* mutant, in which initiation of replication is temperature sensitive. Low concentrations of oxolinic acid, but not coumermycin A_1, reduced thermal inhibition of DNA synthesis rate.

The discovery of DNA gyrase (DNA topoisomerase II [12]) has provided insight into how DNA strands separate during DNA replication in bacteria. By introducing negative supertwists into DNA, gyrase is able to relieve the positive superhelical tension expected to arise from unwinding the DNA helix in a closed, circular DNA molecule. Studies with antibiotics which specifically inhibit gyrase show that the enzyme is responsible for maintaining negative supercoiling in the bacterial chromosome (6). Since these drugs also inhibit DNA synthesis, the implication is that gyrase and negative supercoiling are necessary for DNA replication (see references 4 and 10 for reviews). Three facets of chromosome replication may rely on gyrase: initiation (7, 8, 27), elongation (5), and decatenation (21, 25). Since gyrase also appears to be important for DNA transcription (20, 22, 26, 30, 32, 34, 37, 41) and recombination (14), it is likely that gyrase and supercoiling are important for all aspects of chromosome function involving DNA strand separation.

Gyrase is composed of two types of subunits, A and B, which together bind to DNA, hydrolyze ATP, and introduce negative supertwists (4, 10). The subunits are selectively inactivated by two classes of antibiotics, subunit A by oxolinic and nalidixic acids, and subunit B by coumermycin A_1 and novobiocin. Inhibition of either subunit blocks supertwisting activity. In the absence of ATP hydrolysis, DNA is relaxed by gyrase; relaxation, which must involve DNA breakage and rejoining, is blocked by inactivation of subunit A. Thus, the breakage-rejoining step resides in subunit A. Since novobiocin or coumermycin A_1 blocks ATP hydrolysis and energy transduction, these two activities are functions of subunit B.

Both drugs stop DNA synthesis, but examination of chromosome structure shows that the two drugs have different physiological effects. Coumermycin A_1, but not oxolinic acid, causes a dramatic decrease in DNA superhelicity (6, 29, 33, 36). Moreover, inhibition of DNA synthesis by coumermycin A_1 parallels DNA relaxation (6). Such a correlation cannot be made with oxolinic acid since little or no relaxation occurs with this drug. In addition, a temperature-sensitive host mutation in *gyrA* has no effect on bacteriophage T7 growth at the restrictive temperature, even though phage production is severely inhibited by nalidixic acid (20). Thus, the action of nalidixic acid is not strictly equivalent to inactivation of gyrase, nor does simple drug inactivation of supertwisting activity account for inhibition of DNA synthesis. An additional element not yet explained is the role of topoisomerase II', a relaxing activity composed of subunit A and a fragment of subunit B. Oxolinic acid, but not novobiocin, inhibits this enzyme (1, 11).

We have examined inhibition of DNA synthesis by oxolinic acid and coumermycin A_1 to better understand the differences in their physiological effects. These data are most easily explained if inhibition of DNA synthesis by coumermycin A_1 occurs primarily from loss of supertwists and supertwisting activity, whereas inhibition by oxolinic acid arises from formation of oxolinic acid-gyrase-DNA complexes that block replication fork or DNA movement or both.

TABLE 1. Bacterial strains

E. coli strain	Genotype	Origin (reference)
AB1157	*ara-14 argE3 galK2 his-4 leu-6 lacY1 mtl-1 proA2 rpsL31 supE44 thi-1 thr-1 tsx-33 xyl-5*	A. J. Clark
AB2480	*galK2 his-4 lacY1 proA2 recA13 rpsL31 supE44? tsx-33 uvrA6*	A. J. Clark
AB2924	*ara-14 galK2 his-4 lacY1 lex-1 metB1 mtl-1 proA2 rpsL31 supE44? thr-1 tsx-33 xyl-5*	B. Bachmann (15)
DG75	*ara-3 leu-6 thyA47 rpsL153*	A. Worcel (40)
EE1	*gyrA* (Nal^r) derivative of PC5	This work
JC2926	*recA13* derivative of AB1157	A. J. Clark
JC5547	*recA13 recB21 recC22* derivative of AB1157	A. J. Clark (39)
LL307	*bfe gyrA* (Nal^r) Δ*(lac-pro) supE thi-1*	L. Lindahl
PC2	*dnaC2* derivative of DG75	A. Worcel (2)
PC5	*dnaA5* derivative of DG75	A. Worcel (2)

MATERIALS AND METHODS

Chemicals, reagents, and bacterial strains. Oxolinic acid, a gift from the Warner-Lambert Research Institute, Morris Plains, N.J., was stored at a concentration of 5 mg/ml at pH 10.2 at 4°C. Coumermycin A_1, lot 448, a gift from W. F. Minor, Bristol Laboratories, Syracuse, N.Y., was stored at 10 mg/ml in dimethyl sulfoxide at 4°C. Oxolinic acid and coumermycin A_1, under the storage conditions used, were stable for at least 2 months and 3 weeks, respectively. [*methyl*-^{3}H]thymidine (40 to 60 Ci/mmol) was a product of New England Nuclear Corp., Boston, Mass.

Strains of *Escherichia coli* K-12 were used in all experiments. Genotypes and sources for these strains are listed in Table 1. All strains were grown in M9 minimal salts medium (24) supplemented with amino acids (20 μg/ml) or thymine (40 μg/ml) or both as necessary.

Measurement of DNA synthesis. The rate of DNA synthesis was determined by transferring 0.2 ml from exponentially growing bacterial cultures to tubes containing 1 μCi of [^{3}H]thymidine. After 2 min, incorporation of radioactivity was terminated by addition of 2 ml of cold 5% (wt/vol) trichloroacetic acid. Acid precipitates were collected on GF/A filters (Whatman, Inc., Clifton, N.J.), washed with cold 1 N HCl and then with 95% ethanol, and dried. Acid-precipitable radioactivity measured by liquid scintillation counting was taken as a measure of the rate of DNA synthesis.

RESULTS

Kinetics of inhibition of DNA synthesis by oxolinic acid and coumermycin A_1. Inhibition of DNA synthesis was more rapid with oxolinic acid than with coumermycin A_1, even when the extent of inhibition by oxolinic acid was less than that observed with coumermycin A_1 (Fig. 1). Consequently, biphasic inhibition kinetics could be observed easily with oxolinic acid (Fig. 1 and 2B), whereas coumermycin A_1 typically produced a gradual decline in synthesis rates (Fig. 1).

Oxolinic acid induces *recA*-dependent DNA synthesis. While measuring inhibition kinetics, we observed that partial inhibition of DNA synthesis by low concentrations of coumermycin A_1 or oxolinic acid was followed by a secondary increase in synthesis (Fig. 2). Since transient treatment of bacterial cells with a variety of DNA synthesis inhibitors, including nalidixic acid, leads to induction of *recA*-, *lexA*-dependent DNA synthesis (17–19), it seemed possible that the low drug concentrations shown

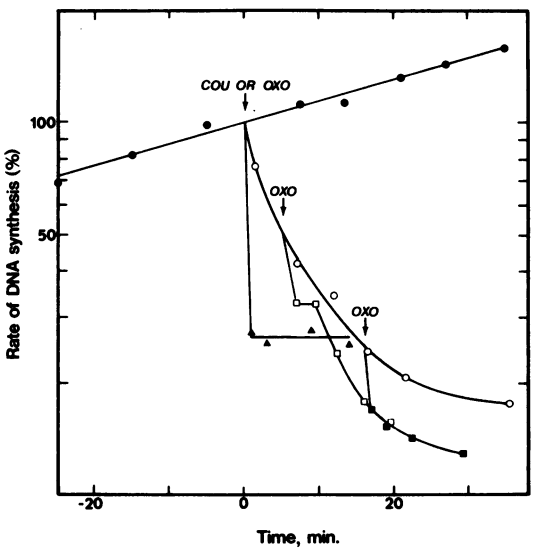

FIG. 1. Inhibition of DNA synthesis by combinations of oxolinic acid (OXO) and coumermycin A_1 (COU). Either coumermycin A_1 (20 μg/ml) (○) or oxolinic acid (0.4 μg/ml) (▲) was added to portions of an exponentially growing culture of *E. coli* JC2926 (*recA*) at zero time. The coumermycin A_1-treated cells were subdivided, and samples were treated with oxolinic acid (0.4 μg/ml) after 5 min (□) or 16 min (■) of incubation in coumermycin A_1, as indicated by the arrows. The rate of DNA synthesis was assayed in each culture at various times as described in the text, and these rates are expressed as percent of the untreated control (●) at zero time.

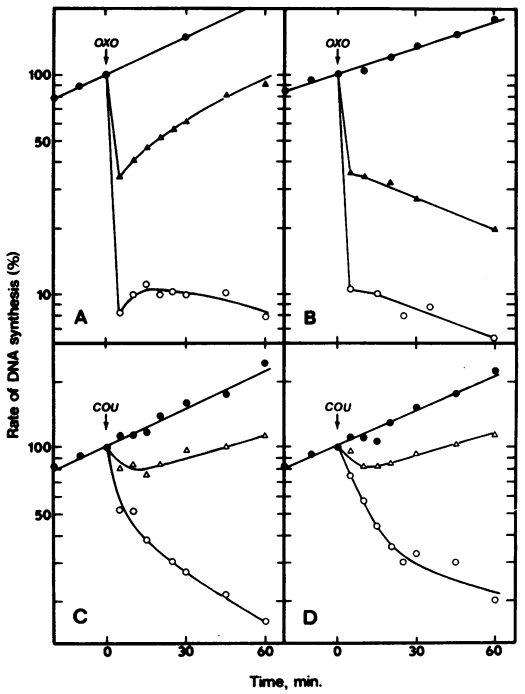

FIG. 2. Effect of *recA* mutation on inhibition of DNA synthesis by oxolinic acid (OXO) and coumermycin A_1 (COU). Rates of DNA synthesis were measured in two strains of *E. coli*, wild type (AB1157; A and C) and *recA uvrA* (AB2480; B and D), treated with oxolinic acid (A and B) or coumermycin A_1 (C and D). Oxolinic acid concentrations were 0.25 μg/ml (▲) or 1.5 μg/ml (○), and coumermycin A_1 concentrations were 5 μg/ml (△) or 15 μg/ml (○). All rates are expressed as a percent of the untreated control (●) at zero time.

in Fig. 2 created a comparable situation. However, analogs of coumermycin A_1 do not induce *recA* (7, 35). Thus, oxolinic acid, but not coumermycin A_1, should induce *recA*-, *lexA*-dependent DNA synthesis. If so, the secondary increase after treatment with oxolinic acid but not coumermycin A_1 should require a functional *recA* gene.

DNA synthesis rates did not exhibit the secondary increase in a *recA13* mutant after addition of oxolinic acid to bacterial cultures (Fig. 2B). Similar measurements with a *lexA* mutant (strains AB2494) showed results identical to those in Fig. 2B. In contrast, inhibition of DNA synthesis by coumermycin A_1 was unaffected by the *recA13* mutation (Fig. 2). Since the data shown in Fig. 2D were obtained with a strain carrying a mutation in *uvrA* as well as *recA*, it appears that neither of these genes affects inhibition of DNA synthesis by coumermycin A_1. We previously reported that inhibition of DNA synthesis by oxolinic acid is also unaffected by the *uvrA* mutation (5).

If blockage of DNA synthesis by oxolinic acid precedes induction of the *recA*-, *lexA*-dependent DNA synthesis, the *recA13* mutation should have no effect on the initial, rapid phase of inhibition. Fig. 2B and 3 show this to be the case.

By using the amount of inhibition occurring during this initial phase as a measure of effective drug concentration, we measured *recA*-dependent DNA synthesis over a wide range of subsaturating drug concentrations. The ratio of the rate of synthesis at 2 h after the addition of oxolinic acid to the rate obtained immediately after completion of the initial, rapid phase of inhibition was calculated. These ratios were then normalized to the ratio found in the appropriate untreated control culture over the same time period to correct for differences in growth rates among the

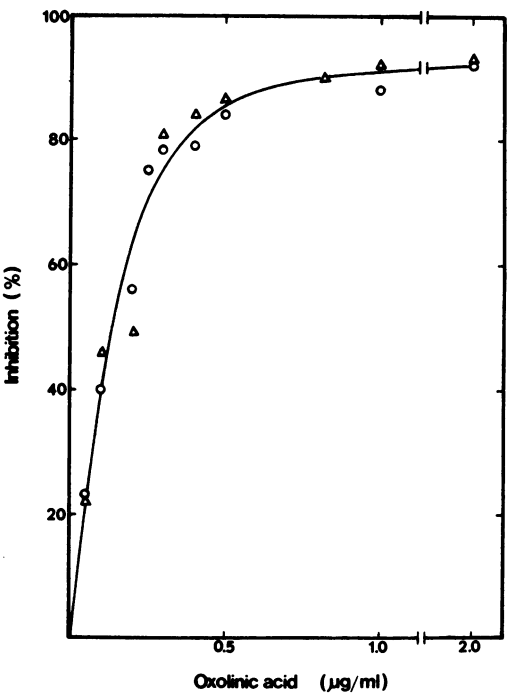

FIG. 3. Effect of oxolinic acid concentration on rapid phase of inhibition of DNA synthesis in $recA^+$ and *recA* strains. Rates of DNA synthesis were measured in two strains of *E. coli*, wild type (AB1157 [△]) and *recA* (JC2926 [○]), at various times after addition of oxolinic acid. The extent of inhibition occurring during the initial, rapid phase of inhibition was determined for each oxolinic acid concentration from plots similar to those shown in Fig. 2A. Generally, the rapid phase was complete within 5 min after addition of the drug. Inhibition is expressed as a percentage of the rate of synthesis in the untreated control at the time of addition of oxolinic acid.

strains tested. The normalized ratios are a measure of secondary DNA synthesis. Fig. 4 shows that, in wild-type strains, the normalized ratios were near unity over a wide range of effective drug concentrations. In contrast, normalized ratios were below unity for the *recA13* strain, and they declined as the effective drug concentration increased. The closed circles in Fig. 4 show that the *recA recB recC* triple mutant responded to oxolinic acid much like the *recA13* strain (open circles, Fig. 4), indicating that the decline in synthesis rates observed in the *recA* strain is not reversed by mutations inactivating the *recBC* nuclease (3, 9, 16, 23, 31, 39).

Oxolinic acid reduces thermal inhibition of DNA synthesis in a *dnaA*(Ts) mutant. As pointed out above, subsaturating doses of oxolinic acid caused DNA synthesis rates to drop quickly, but within a few minutes wild-type strains showed a striking increase in DNA synthesis. To determine if this increase in synthesis involves initiation of new rounds of replication, we treated cultures of temperature-sensitive initiation mutants with oxolinic acid and measured synthesis rates after shifting the cultures to nonpermissive temperatures. In the case of a *dnaC* mutation (PC2 [2]), incubation at the nonpermissive temperature blocked the increase in DNA synthesis which characteristically follows initial inhibition by the drug (data not shown). By this criterion, it appears that induction of DNA synthesis by oxolinic acid involves initiation of replication.

A *dnaA*(Ts) initiation mutant (PC5), however, behaved differently (Fig. 5). The open circles in Fig. 5A show the normal, gradual decline in synthesis rate when the incubation temperature of this mutant was raised to 40.5°C, presumably because new rounds of replication were unable to initiate (the brief increase in synthesis rate immediately after the temperature shift probably reflects accelerated rates of replication fork movement). Addition of subsaturating concentrations of oxolinic acid 10 min after shifting the bacterial culture to 40.5°C sharply reduced the rate of synthesis, but after 30 min, the decline in synthesis rate stopped (Fig. 5A). A similar phenomenon was observed when oxolinic acid was added to the culture before increasing the incubation temperature (Fig. 5B). Oxolinic acid induced the characteristic rapid inhibition of DNA synthesis at the permissive temperature, but when the culture was shifted to the nonpermissive temperature, the rate of synthesis increased rather than declining further. This increase was possibly due to faster fork movement at the higher temperature and was also observed with a wild-type strain (data not shown). In contrast, coumermycin A_1 failed to affect the inhibitory action of the *dnaA*(Ts) mutation (Fig. 5C and D).

To establish that the target of oxolinic acid is gyrase subunit A in the *dnaA*(Ts) mutant, we constructed a *dnaA*(Ts) *gyrA* (oxolinic acid-resistant) double mutant by P1-mediated transduction (38) and treated it with oxolinic acid. The drug had no effect on thermal inhibition of DNA synthesis in this strain (data not shown).

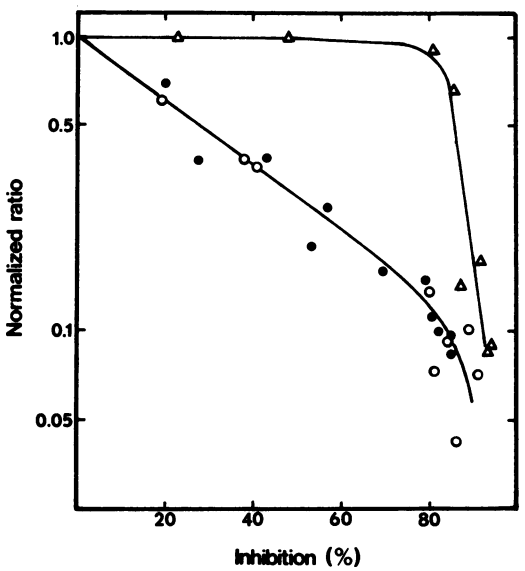

FIG. 4. Changes in residual DNA synthesis rates after treatment with oxolinic acid. Exponentially growing wild type AB1157 (△), *recA* JC2926 (○), and *recA recB recC* JC5547 (●) were treated with various concentrations of oxolinic acid, and DNA synthesis rates were measured to generate plots similar to those shown in Fig. 2. The ratio of the rate of synthesis 2 h after addition of oxolinic acid to the rate immediately after completion of the initial inhibition was then calculated for each experiment. These ratios were then normalized to the ratio found in the appropriate untreated control culture over the same time period to correct for differences in growth rates among the strains tested. The abscissa of the figure represents the fraction of DNA synthesis inhibited by oxolinic acid during the initial inhibitory phase.

DISCUSSION

Antibiotics which selectively affect different subunits of gyrase have different physiological consequences in bacterial cells. The most striking feature of inactivation by oxolinic acid is the rapid inhibition of DNA synthesis that occurs even when inhibition is only partial (5, 36; Fig. 2). Shortly after partial inhibition has occurred, *recA*-, *lexA*-dependent DNA synthesis appears and persists for several hours (Fig. 2A and reference 4). At higher drug doses, DNA synthesis is inhibited to greater than 95% (Fig. 3), *recA-lexA*-dependent DNA synthesis is absent (Fig. 4), and *recA* is induced (7, 13). In contrast, the prominent characteristic of inactivation of gy-

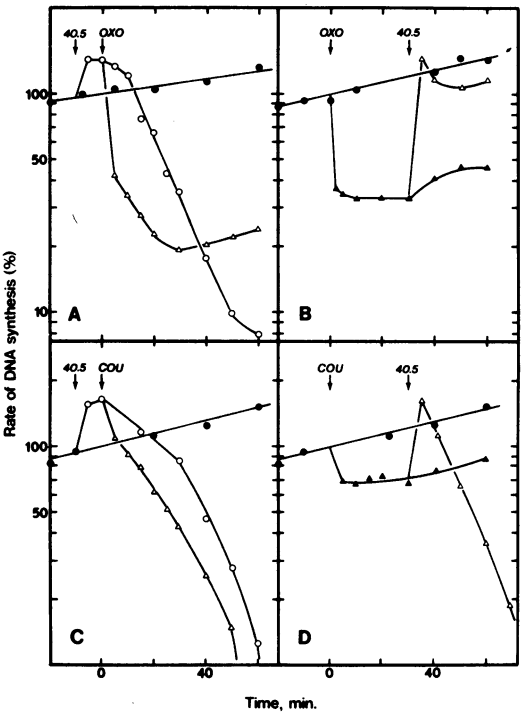

FIG. 5. Effect of oxolinic acid (OXO) and coumermycin A_1 (COU) on thermal inhibition of DNA synthesis in *dnaA*(Ts) mutants. Exponentially growing cultures of *E. coli* PC5 (*dnaA*[Ts]) were shifted from 29 to 40.5°C at the times indicated, and rates of DNA synthesis were measured (○). After 10 min of incubation at 40.5°C, the cultures were treated with oxolinic acid or coumermycin A_1. Rates of DNA synthesis in the presence of these drugs at 40.5°C (△) are shown in A and C for oxolinic acid (0.35 μg/ml) and coumermycin A_1 (5 μg/ml), respectively. Rates of synthesis when the two drugs are added 30 min before the shift from 29 to 40.5°C are shown in B and D for oxolinic acid (0.2 μg/ml, and coumermycin A_1 (5 μg/ml), respectively. Symbols represent synthesis in the presence of each drug at 29°C (▲) or after the temperature shift to 40.5°C (△). All rates of synthesis are expressed as a percentage of the untreated control (●) at zero time. Each experiment was repeated at least three times, and the replicates were qualitatively identical. Thermal inhibition of DNA synthesis was measured nine times; the average time to reach 50% inhibition was 33 ± 3 (standard deviation) min.

rase by coumermycin A_1 is the correlation between DNA relaxation and inhibition of DNA synthesis (6). The gradual inhibition kinetics produced by coumermycin A_1 differ qualitatively from the biphasic kinetics obtained with oxolinic acid (Fig. 1). Neither *recA* (7, 35) nor *recA*-, *lexA*-dependent DNA synthesis (Fig. 2) is induced by coumermycin A_1.

Whereas DNA synthesis after low doses of oxolinic acid is reduced in *recA* and *lexA* mutants, little or no induction of *recA* occurs in wild-type strains at these low drug concentrations (below 0.5 μg/ml; data not shown). Thus, additional studies are necessary to determine if the *recA*-, *lexA*-dependent DNA synthesis requires induction of *recA* or if it is simply very sensitive to constitutive levels of the *recA* protein.

In vitro studies provide some assistance in interpreting the physiological effects of oxolinic acid. The drug appears to trap a reaction intermediate in the supercoiling reaction, producing a drug-gyrase complex. These complexes are detected by exposure to sodium dodecyl sulfate, whereupon DNA cleavage occurs and the *gyrA* gene product becomes covalently linked to DNA (4, 10, 28, 36). In vivo these complexes could act as barriers to replication fork or DNA movement or both, rapidly inhibiting DNA synthesis and inducing *recA*. Comparable complexes have not been observed with coumermycin A_1, suggesting that inhibition of DNA synthesis by coumermycin A_1 and oxolinic acid differ qualitatively. Inhibition of DNA synthesis by coumermycin A_1 may arise from chromosome relaxation as well as loss of supertwisting activity.

In vitro studies, however, add little insight into how oxolinic acid reduces thermal inhibition of DNA synthesis in a *dnaA*(Ts) mutant (Fig. 5). Genetic studies indicate that the phenomenon is mediated by the *gyrA* gene product since it was absent in a *gyrA*(Oxo^r) derivative of the *dnaA* mutant. Moreover, it appears to be restricted to *dnaA*; oxolinic acid had no effect on thermal inhibition in a *dnaC*(Ts) mutant. One explanation is that oxolinic acid perturbs an interaction between gyrase and the *dnaA* gene product (7, 8, 27). Alternatively, the results may be another reflection of differences between the *dnaA5* and *dnaC* mutations in establishment of *recA*-, *lexA*-dependent DNA synthesis by oxolinic and nalidixic acids (18). Other, less interesting interpretations arise from ambiguities in kinetic experiments such as these. First, to detect small differences in DNA synthesis rates, it was necessary to measure incorporation of radioactive precursors rather than changes in DNA mass. Isotope incorporation rates can be affected by changes in precursor pool sizes. Second, adequate data concerning drug uptake rates and clearing times are unavailable. Consequently, kinetic studies serve primarily as guides for more definitive genetic and in vitro biochemical approaches.

In conclusion, oxolinic acid and coumermycin A_1 specifically affect the *gyrA* and *gyrB* gene products, respectively, and in so doing they produce different perturbations of bacterial chromosome structure. In the case of oxolinic acid, the formation of drug-subunit A-DNA

complexes appears to lead to a set of *recA*-, *lexA*-dependent secondary effects that do not arise from chromosome relaxation by coumermycin A_1.

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