# Characterization of a Cinodine-resistant Mutant of *Escherichia coli*

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Bacterial topoisomerase II (DNA gyrase) catalyzes topological conversions of DNA molecules that are essential to bacterial growth[1]. DNA gyrase is the target of two known classes of antibiotics: the coumarins, natural products that target the B subunit of the enzyme[2,3], and the quinolones, synthetic products that target the A subunit of the enzyme and probably interfere with the DNA rejoining step of the gyrase-mediated DNA strand-passing reaction[4~7]. Two other types of antibiotics, clerocidin[8], and cinodine[9], have recently been shown to inhibit bacterial DNA gyrase. Clerocidin was shown to interfere with gyrase-mediated DNA cleavage[8]. An *Escherichia coli* mutation conferring resistance to clerocidin was found to map in the *gyrA* gene[8]. Cinodine, a glycocinnamoylspermidine antibiotic (Fig. 1), is known to bind to DNA and to interfere with DNA synthesis[10]. However, at lower concentrations, it was found to inhibit specifically the supercoiling reaction of DNA gyrase *in vitro*[9]. In this communication the properties of a cinodine-resistant mutant strain of *Escherichia coli* are described. The data presented strongly suggest that at low drug concentrations, DNA gyrase is the *in vivo* target of cinodine.

## Derivation and Phenotype of Cinodine-resistant Mutant MSO101

Many unsuccessful attempts have been made previously in this and other laboratories to isolate cinodine-resistant mutants of *E. coli*. More recently, however, I isolated four spontaneous cinodine-resistant mutants. These were picked from a zone of inhibition resulting from a 1 $\mu$g spot of cinodine (mixture of the $\gamma_1$ and $\gamma_2$ forms) on a lawn of *E. coli* strain KNK453, grown at 28°C on an LB[11] plate. The mutant colonies were near the outer edge of the zone. Strain KNK453 is a temperature-sensitive *nal* derivative of *E. coli* C[12]. The mutant colonies appeared after 4 to 5 days of incubation. One mutant, strain MSO101, was characterized, and exhibited low-level resistance to cinodine *in vivo*.

Strain MSO101 grew with a doubling time of 70 minutes at 28°C in the absence of cinodine, in contrast to 60 minutes for the parent strain. Table 1 shows the sensitivity of strain MSO101 to cinodine and susceptibility to several other antibacterial agents. In this regard, MSO101 differed from the parent and wild type strains only in sensitivity to cinodine and to nalidixic acid (a known inhibitor of DNA gyrase subunit A). MSO101 was four-fold more resistant to cinodine than strain KNK453, and more sensitive to nalidixic acid, suggesting that the new mutation in MSO101 resulted in an alteration in DNA gyrase.

## DNA Gyrase from MSO101 was More Resistant to Cinodine

Extracts of DNA gyrase from strains MSO101 and the parent KNK453 were assayed *in vitro* for the ability to convert relaxed plasmid pBR322 DNA to the supercoiled form in the presence and absence of cinodine. Cell extracts were prepared from 500 ml of cells grown to late log phase in LB medium at 28°C. Cells were

Fig. 1. Structures of the $\beta$, $\gamma_1$, and $\gamma_2$ forms of cinodine.

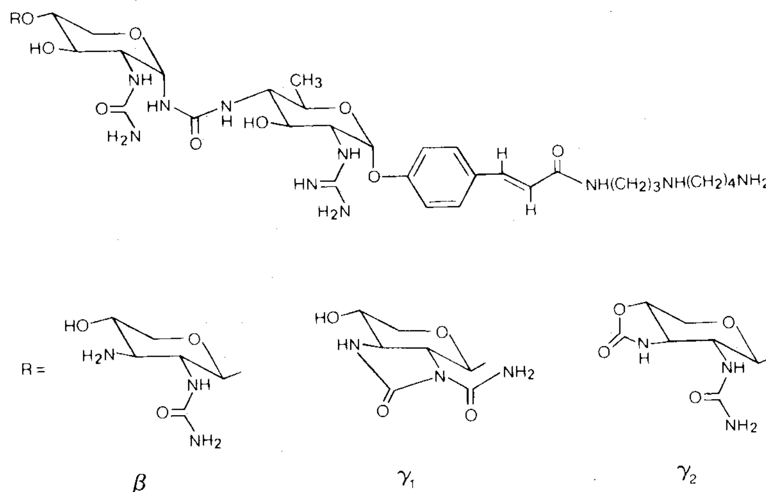


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Table 1. MIC's of various antibiotics for strains *E. coli* C, KNK453, and MSO101[a].

| Antibiotic | Target | MIC (μg/ml) | | |
|---|---|---|---|---|
| | | *E. coli* C | KNK453 | MSO101 |
| Cinodine | | <0.05 | 0.10 | 0.40 |
| Nalidixic acid | Gyrase A subunit | 1.00 | 50.00 | 10.00 |
| Coumermycin | Gyrase B subunit | 15.00 | 40.00 | 40.00 |
| Ampicillin | Cell wall | 15.00 | 15.00 | 15.00 |
| Chloramphenicol | Protein synthesis | 2.50 | 5.00 | 2.50 |
| Streptomycin | Protein synthesis | 50.00 | 50.00 | 50.00 |
| Rifampicin | RNA polymerase | 25.00 | 25.00 | 25.00 |

[a] Cells were grown at 28°C in LB medium containing various concentrations of the indicated antibiotics.

Table 2. *In vitro* activity of DNA gyrase extracts from strains MSO101 and KNK453 in the presence of cinodine[a].

| Strain | Assay mixture | Fraction of DNA converted from relaxed to supercoiled form |
|---|---|---|
| KNK453 | | |
| | Complete, no drug | 1.00[b] |
| | Complete, +25 μg novobiocin/ml | <0.02 |
| | Complete, +1 μg cinodine/ml | 0.46 |
| | Complete, +2 μg cinodine/ml | <0.02 |
| | Complete, +3 μg cinodine/ml | <0.02 |
| | Complete, +4 μg cinodine/ml | <0.02 |
| | No enzyme, no drug | <0.02 |
| MSO101 | | |
| | Complete, no drug | 1.00[b] |
| | Complete, +25 μg novobiocin/ml | <0.01 |
| | Complete, +1 μg cinodine/ml | 0.99 |
| | Complete, +2 μg cinodine/ml | 0.94 |
| | Complete, +3 μg cinodine/ml | 0.88 |
| | Complete, +4 μg cinodine/ml | 1.00 |
| | Complete, +5 μg cinodine/ml | <0.01 |

[a] The supercoiling reaction of DNA gyrase was carried out at 30°C by the method described previously, using 0.5 μg of relaxed plasmid pBR322 as the substrate[9]. Assay tubes contained 5 μg of protein extract. Reaction products were analyzed by the electrophoretic assay method[14] and quantitated by densitometry[9].

[b] The fraction of supercoiled DNA produced by DNA gyrase in the absence of inhibitor is designated as 1.00.

pelleted, resuspended in 5 ml 0.1 M $KH_2PO_4$ buffer (pH 7.6) containing 0.1 mM dithiothreitol, sonicated for 3 minutes in 1 minute pulses at 0°C, and centrifuged at 40,000 × *g* for 30 minutes at 4°C. The supernatant fluid constituted cell extract. Protein concentration was determined by the method of LOWRY[13]. Supercoiling activity of gyrase was measured by the standard electrophoretic assay method[14]. The assay used to measure inhibition of supercoiling by novobiocin and cinodine, and quantitation by means of densitometry, were described in detail previously[9]. The identical assay was used here, except that cell extract was used in place of purified *Micrococcus luteus* DNA gyrase. Results (Table 2) show that for equal amounts of protein, the gyrase activity of MSO101 was four-fold more resistant to cinodine than the gyrase activity of strain KNK453. This result is likely due to either an increased level of gyrase in the mutant strain, or to a genetic alteration in the gyrase structural gene.

## Discussion

Results show that a cinodine-resistant mutant of *E. coli* was approximately 4-fold more resistant to the drug *in vivo* than the parent strain, and that the mutation resulted in an alteration in DNA gyrase supercoiling activity. The apparent occurrence of cinodine-resistant mutants is quite rare. It was shown previously[9] that the inhibitory effect of cinodine on supercoiling occurred at a much lower concentration than that required to bind to DNA and alter its mobility. The two distinct activities of cinodine are likely to contribute to the difficulty in isolating cinodine-resistant mutants. *In vitro* results have shown that supercoiling by wt DNA gyrase was inhibited at cinodine concentrations of 0.1 to 0.2 times the concentration required to inhibit DNA synthesis[9]. Thus, mutations conferring resistance to cinodine may be detectable only by selecting for low-level cinodine-resistant colonies, since selection for high-level resistance

would require resistance to both the gyrase- and the DNA synthesis-inhibiting activities of cinodine. This would explain why resistant mutants appeared in a zone of inhibition, which contains a range of drug concentrations.

Another explanation for the difficulty in isolating *E. coli* cinodine-resistant mutants may involve an interaction with topoisomerase IV. This enzyme has significant homology to DNA gyrase, catalyzes relaxation of supercoiled DNA, and is inhibited by known inhibitors of the A and B subunits of DNA gyrase[15]. Evidence indicates that topoisomerase IV is an essential enzyme[15] although overproduction of DNA gyrase can complement conditional lethal mutations in the genes encoding the topoisomerase IV subunits[16]. If cinodine also inhibits topoisomerase IV, high-level cinodine-resistance might require mutations in multiple genes.

Thus far cinodine-resistant mutants have been isolated only from *E. coli* encoding a preexisting mutation in *gyrA*. This suggests the possibility that the *gyrA* or other unknown mutation (perhaps acquired by strain KNK453 during passage) may be a prerequisite for identification of a cinodine-resistant mutant.

Although cinodine inhibits DNA gyrase at a lower drug concentration than that required to inhibit DNA synthesis, the lethal event in cinodine inhibition of growth is not known. Recently, it was shown that DNA gyrase-quinolone complexes to DNA and blocks transcription in *E. coli*[17]. The authors postulated that this blockage may contribute to the lethality of quinolones, since much lower levels of quinolone are needed to inhibit growth than to inhibit supercoiling. Such secondary phenomena may also occur with cinodine, as much lower concentrations of the drug are required to inhibit growth than to inhibit supercoiling *in vitro*, or DNA synthesis *in vivo*[10].

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