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DNA GYRASE (TOPOISOMERASE II) FROM PSEUDOMONAS AERUGINOSA

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DNA gyrase (Topoisomerase II) has been purified from Pseudomonas aeruginosa strain PAO. This enzyme is inhibited by novobiocin and nalidixic acid. DNA gyrase from P. aeruginoisa is resistant to a much higher level of nalidixic acid than is Escherichia coli DNA gyrase. This increased level of resistance may explain, at least in part, the higher levels of natural resistance exhibited by P. aeruginosa toward nalidixic acid.

Naturally occurring strains of Pseudomonas aeruginosa are resistant to higher concentrations of the drug nalidixic acid than most other Gram-negative bacterial species (1). Typical minimal inhibitory concentrations (MICs) of P. aeruginosa for nalidixic acid are 128 to >256 μg/ml while MICs for other Gram-negative species range from 4 to 8 μg/ml (1). Studies by Gellert, et al. (2) and Sugino, et al. (3) have shown that the mechanism of action of nalidixic acid in Escherichia coli is to inhibit the enzyme DNA gyrase (Topoisomersase II), and that resistant strains of E. coli contain a resistant form of this enzyme (2, 4). This paper demonstrates the presence in P. aeruginosa of DNA gyrase which is more resistant to nalidixic acid than is the enzyme from E. coli.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions. For this study, strain RM40 (5), a derivative of strain PAO, was used. This strain has an MIC for nalidixic acid of 175 μg/ml on Mueller-Hinton Medium. For the isolation of DNA gyrase, cultures were grown in Luria Broth as described by Scurlock and Miller (6).

Purification of DNA Gyrase. Cultures were grown, harvested, and lysed by the method of Scurlock and Miller (6). This procedure yielded 16 to 20 g (wet

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weight) of cells. All subsequent operations were carried out at 4° C. Cell debris and DNA were removed by centrifugation for 30 min at 35,000 X g. The supernatant fluid was decanted and treated with a 5% (w/v) solution of streptomycin sulphate prepared in water. One milliliter of the streptomycin solution was added for each 425 optical density units at 260 nm present. The mixture was stirred for 20 min, and the precipitate which formed was separated by centrifugation for 20 min at 27,000 X g. The precipitate was discarded, and the supernatant fluid was dialyzed overnight against 1.5 liters of 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol, 0.1 mM EDTA, and 10% (w/v) glycerol. This fraction was made 30% (w/v) in glycerol and layered onto a DEAE-cellulose column (2.5 x 12.5 cm) which was equilibrated with 50mM Tris-HCl (pH 8.0) containing 0.1 mM dithiothreitol, 0.1mM EDTA, and 30% (w/v) glycerol (Buffer A). The sample was followed by a 35 ml wash of Buffer A and a 300 ml gradient of 0.05 to 0.3 M NaCl prepared in Buffer A. The fractions which eluted between 0.1 and 0.2 M NaCl were pooled. Ammonium sulphate was then added to the pooled fractions to a final concentration of 70% (w/v). The precipitate which formed upon stirring for 20 min was collected by centrifugation and dialyzed overnight against 1 liter of 0.03 M potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol, 0.1 mM EDTA, and 30% (w/v) glycerol (Buffer B).

The dialyzed solution was layered onto a column of Sephadex G-200 (1.5 x 48 cm) which had been equilibrated in Buffer B. The column was eluted with Buffer B. Fractions showing gyrase activity were pooled, concentrated with a Millipore Submersible Concentrator, and layered onto a hydroxyapatite column (0.5 x 5 cm) which had been equilibrated in 10 mM potassium phosphate buffer (pH 7.0) containing 6 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (w/v) glycerol (Buffer C). The column was eluted with a 0.1 to 0.7 M phosphate buffer solution containing 6 mM 2-mercaptoethanol, 1 mM EDTA and 10% glycerol. Each fraction (1.0 ml) was dialyzed against Buffer C and tested for gyrase activity. Peak activity eluted between 320 and 360 mM phosphate. This fraction was stored at -20° C until used.

DNA Gyrase Assay. *P. aeruginosa* DNA gyrase activity was determined in a reaction which measured the conversion of relaxed, closed-circular DNA to the supercoiled form as demonstrated by agarose gel electrophoresis. The reaction mixture (100 µl) contained 35 mM Tris-HCl (pH 7.5), 6 mM MgCl , 1.5 mM dithiothreitol, 1.5 mM spermidine-HCl, 1.5 mM ATP, 5 µg bovine serum albumin, 0.6 µg of relaxed, closed-circular DNA, and an enzyme sample of various concentrations. The DNA in the reaction was either relaxed, closed-circular ϕX174 (RF1) DNA or relaxed, closed-circular ColE1 DNA. The ϕX174 (RF1) DNA was purchased from New England Biolabs while the ColE1 DNA was prepared from *E. coli* strain JC411(ColE1) as described by Tanaka and Weisblum (7). Relaxed, closed-circular forms of the DNAs were prepared from supercoiled DNAs using a nicking-closing extract prepared from chicken reticulocytes as described by Gellert *et al.* (8). The reaction mixture was incubated at 37° C for 60 min. The reaction was stopped by addition of 5 µl of 0.25 M EDTA, and the solution was extracted with an equal volume of chloroform-isoamyl alcohol (24:1 v/v). The aqueous phase was added to 5 µl of a solution of 5% sarkosyl, 25% glycerol, and 0.025% bromphenol blue, loaded onto a 0.8% agarose gel and electrophoresed at 65 volts for 16 h (9). The gel was then stained with a solution of 0.5 µg ethidium bromide/ml of water for 30 min, destained for 30 min in water, and photographed with a Polaroid MP-4 camera using Polaroid type 57 film and UV filter (9). One unit of DNA gyrase converts 0.1 µg of relaxed DNA to the supercoiled form in 30 min at 37°C.

In the experiments examining the inhibition of *P. aeruginosa* DNA gyrase by various drugs, gyrase activity was quantitated by scanning negatives of the photographs prepared from the agarose gels with a Kratos-Schoeffel SD3000 Spectrodensitometer equipped with a Hewlett-Packard 3390A integrator as described by Sugino and Bott (10).

Determination of Molecular Weight by Glycerol Gradient Centrifugation. Linear glycerol gradients (20 to 40%) prepared in 30 mM potassium phosphate buffer (pH 7) were centrifuged in a Beckman SW60 swinging-bucket rotor at 50,000 rpm (260,000 X g) for 12 h in a Beckman L5-75 ultracentrifuge. The gradients were divided into 30 fractions and assayed for DNA gyrase activity. Molecular weight of P. aeruginosa DNA gyrase was determined from the relative sedimentation velocity using the fraction showing the highest DNA gyrase activity (6, 11). Aldolase, catalase, and thyroglobulin were used as sedimentation standards.

Protein Determinations. Protein determinations were by the method of Lowry et al. (12) using BSA as a standard. Concentrations below 0.05 mg/ml were measured by ultraviolet absorbancy at 280 nm.

RESULTS AND DISCUSSION

Preparations of purified P. aeruginosa DNA gyrase had a specific activity of 15,000 units/mg protein and had been purified a minimum of 1200-fold (Table 1). Difficulty in accurately determining the activity of DNA gyrase in crude extracts prevented an accurate determination of the purification and yield. Mixing of purified fractions with material from earlier fractions indicated that this activity was due to an inhibitor which was purified away from the DNA gyrase on the DEAE-cellulose column. Whether this is a specific or a non-specific (e.g. an endonuclease) inhibitor has not been determined. The preparation was unstable and activity could only be retained for 2-3 weeks when stored at -20°C. Figure 1 demonstrated P. aeruginosa DNA gyrase activity on relaxed, closed-circular ϕX174 DNA. The enzyme requires ATP and magnesium for supercoiling activity and is inactivated by heating to 70°C for 15 min. The molecular weight of P. aeruginosa DNA gyrase was estimated to be

Table 1. Purification of Pseudomonas DNA Gyrase.

	FRACTION	VOLUME (ml)	PROTEIN (mg/ml)	ACTIVITY (Units/mg protein)
I.	Cleared Lysate	29	9.8	ND[a]
II.	Streptomycin	29	6.0	ND
III.	DEAE-Cellulose	6	7.1	13
IV.	Sephadex G-200	3	2.7	50
V.	Hydroxyapatite	2	0.01	15,000

[a]ND: DNA gyrase activity was not detectable in these fractions (see text).

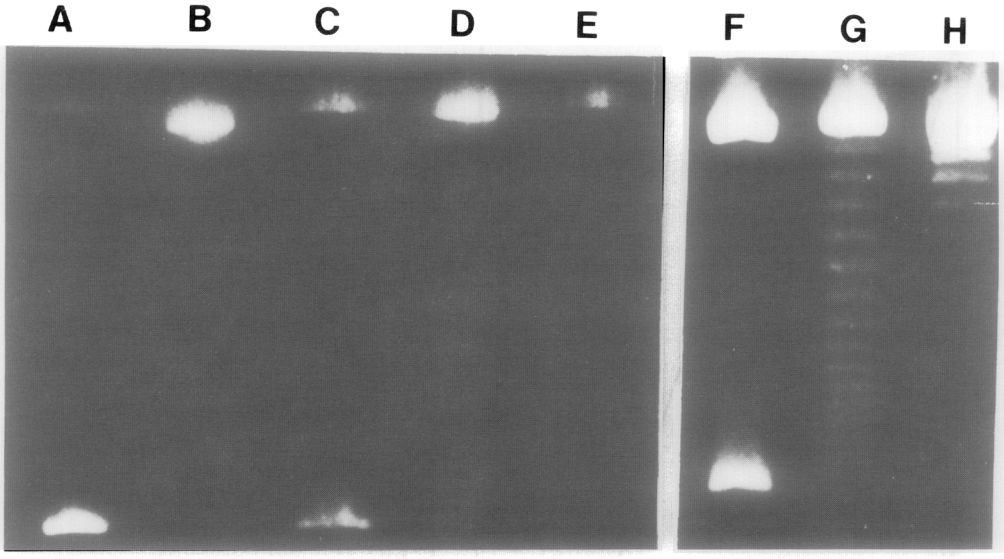

Figure 1. **Assay of P. aeruginosa DNA gyraseactivity under different conditions.** (A) Supercoiled ϕX174 (RF1) DNA (lower band) containing some open-circular DNA (upper band). (B) Relaxed, double-stranded ϕX174 DNA. (C) Relaxed, double-stranded ϕX174 DNA incubated with 30 μl of purified gyrase under standard reaction conditions. (D-H) Relaxed, double-stranded ϕX174 DNA incubated with 30 μl of purified gyrase for 60 min under the following conditions: (D) ATP omitted; (E) Magnesium omitted; (F) Standard reaction conditions; (G) Nalidixic acid added (500 μg/ml); (H) Novobiocin added (1 μg/ml).

3.6±0.3 x 10^5 by sedimentation through a 20 to 40% (w/v) glycerol gradient (6, 11).

The sensitivity of P. aeruginosa DNA gyrase to nalidixic acid and novobiocin was investigated. Samples of purified enzyme were incubated in the standard reaction mixture containing various concentrations of one or the other of these inhibitors. Escherichia coli DNA gyrase was purified to the same specific activity from strain HN356 (recB21, obtained from M. Gellert) by the method of Gellert et al. (8). These preparations were used for comparative purposes. P. aeruginosa DNA gyrase was found to be inhibited by both of these antibiotics (Figure 1). While the sensitivity of P. aeruginosa and E. coli DNA gyrases to novobiocin is similar, significantly higher concentrations of nalidixic acid are required to inhibit the P. aeruginosa enzyme than the E. coli enzyme (Figure 2). The concentration of nalidixic acid necessary to inhibit 50% of the activity of P. aeruginosa gyrase, 600 μg/ml, is approximately 3-fold higher than the MIC determined for this strain

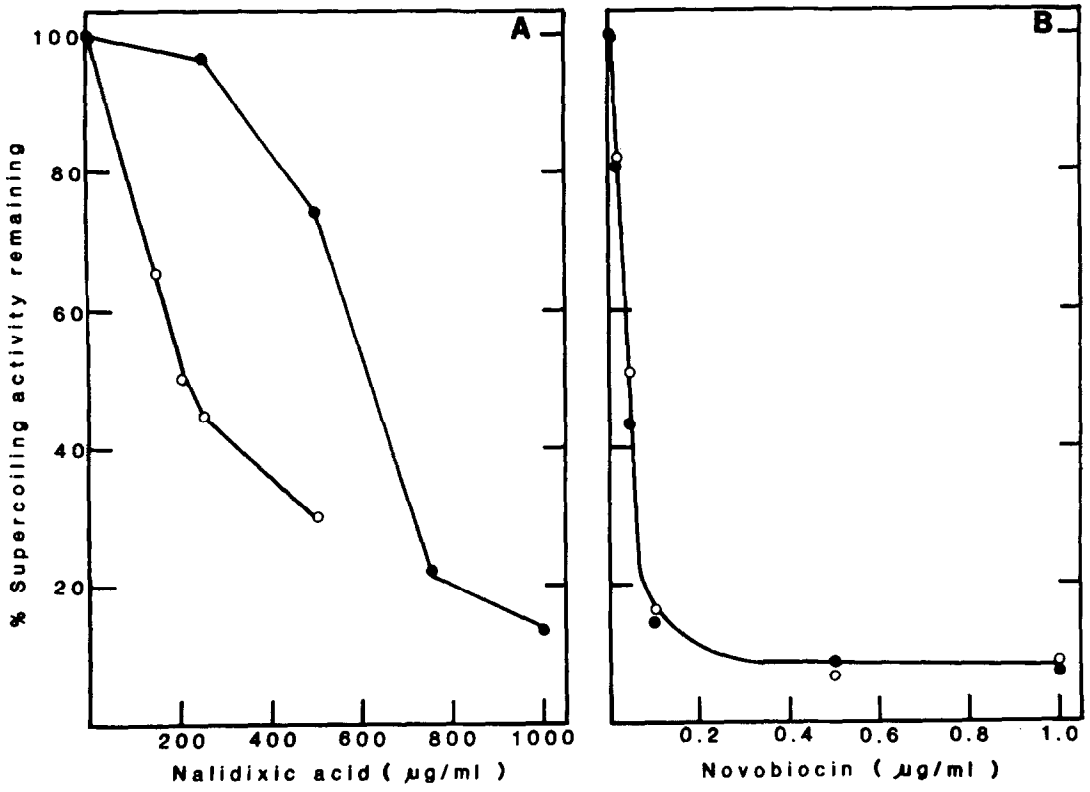

Figure 2. Sensitivity of DNA gyrase to nalidixic acid and novobiocin. Standard supercoiling assays containing relaxed φX174 DNA and different concentrations of nalidixic acid or novobiocin were carried out. Supercoiling activity was quantitated by tracing photographic negatives of the stained gels as described by Sugino and Bott (10). (A) Sensitivity of DNA gyrase to nalidixic acid; (B) Sensitivity of DNA gyrase to novobiocin. (●) P. aeruginosa DNA gyrase; (○) E. coli DNA gyrase.

(175 µg/ml). The E. coli gyrase was 50% inhibited by a concentration of 200 µg/ml (3 and this study) which is 50-fold higher than the MIC (4 µg/ml) of the E. coli strain.

Engle et al. (13) have suggested that the in vitro inhibitory action of nalidixic and oxolinic acids in E. coli is to form drug-gyrase-DNA complexes which inhibit replication-fork migration. They have shown that this inhibition of DNA replication can be relieved by a recA-dependent repair mechanism. Certainly this inhibitory mechanism would be extremely sensitive to the drug and a single DNA-associated complex could inhibit cell growth. This mechanism is consistent with inhibition of growth at a drug concentration which would inhibit only a small fraction of the enzyme activity as is seen in E. coli (the MIC of this organism corresponds to a concentration of nalicixic acid which in

vitro inactivates approximately 5% of the gyrase activity). While the *P. aeruginosa* DNA gyrase requires greater concentrations of nalidixic acid for 50% inhibition, the MIC of this organism is also much higher. A concentration of drug which *in vitro* inactivates approximately 5 to 10% of the gyrase activity is equilivalent to the *in vivo* MIC of this organism. This indicates that the mechanism of *in vivo* inhibition of growth in *P. aeruginosa* is also extremely sensitive to drug-gyrase interaction and suggests that the mechansim may be similar to that proposed by Engle, *et al.* (13) for *E. coli* gyrase.

The heightened resistance in *P. aeruginosa* may simply be due to a higher K_i for nalidixic acid inhibition of *P. aeruginosa* DNA gyrase. This would imply that the drug-gyrase-DNA complexes would be more easily dissociated. In this context, we have not been able to demonstrate drug-gyrase-DNA complex formation with *P. aeruginosa* gyrase *in vitro* as measured by sodium dodecyl sulfate-induced cleavage of the DNA at the site of complex attachment (14, 15, 16, 17,18) under conditions where complex formation with the *E. coli* DNA gyrase was detected by DNA cleavage. Hence, it appears probable that the stability of the drug-gyrase-DNA complex is much reduced in *P. aeruginosa*, and this instability leads to the increased resistance of this species and its DNA gyrase to nalidixic acid both *in vitro* and *in vivo*.

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