To the Graduate Council:

I am submitting herewith a dissertation written by Ted Ray Scurlock entitled "Identification of a New Deoxribonuclease and DNA Gyrase in Pseudomonas aeruginosa Strain PAO." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Robert V. Miller, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

IDENTIFICATION OF A NEW DEOXYRIBONUCLEASE AND DNA GYRASE IN *PSEUDOMONAS AERUGINOSA* STRAIN PAO

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Ted Ray Scurlock

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ABSTRACT

Deoxyribonucleases are known to be involved in genetic recombination in Escherichia coli. Identifying deoxyribonucleases in Pseudomonas aeruginosa and comparing them to the deoxyribonucleases in E. coli will help understand genetic recombination in P. aeruginosa.

A new deoxyribonuclease, PaeExoIX, has been purified to electrophoretic homogeneity from extracts of P. aeruginosa by using two DEAE-cellulose columns, a Sepharose 6B column, and a glycerol gradient. This enzyme, which was active in the presence of EDTA, was equally efficient in hydrolyzing native and heat-denatured DNA to acid-soluble products. The enzyme was partially or totally inhibited by the presence of several divalent cations. Optimal activity was demonstrated between pH 8.0 and 9.0. The active protein has a molecular weight of $1.6 \pm 0.1 \times 10^5$ and is composed of two nonidentical polypeptides with molecular weights of 78,000 and 69,000. Preliminary data indicate that both the 3' end and the 5' end of DNA are rapidly degraded by this enzyme.

The data indicate that this enzyme has several unique biochemical properties when compared to other deoxyribonucleases identified in other bacteria. However, the enzyme has activities which are similar to those of several deoxyribonucleases which are involved in genetic recombination in other bacteria.

The second part of this study is a report of DNA gyrase in P. aeruginosa. This enzyme catalyzes the supercoiling of DNA in the presence of ATP. Its activity was detected by using a relaxed closed-circular DNA substrate in the reaction mixture followed by analysis with agarose gel electrophoresis. DNA gyrase has also been identified in E. coli, Micrococcus luteus, and Bacillus subtilis by other investigators. DNA gyrase from these species is inhibited by the drugs nalidixic acid and novobiocin.

DNA gyrase from P. aeruginosa, like DNA gyrase identified in other bacteria, requires ATP and was inhibited by the drugs novobiocin and nalidixic acid. The native enzyme has a molecular weight of 360,000 when estimated by Sephadex G-200 chromatography. Double-stranded cleavage or relaxing activity could not be demonstrated in P. aeruginosa DNA gyrase as is found in E. coli gyrase. However, a relaxing activity was separated from P. aeruginosa DNA gyrase with a Sephadex G-200 column. Therefore, P. aeruginosa does have a DNA gyrase type enzyme, but there are several differences in its properties when compared with DNA gyrase from other bacteria.

TABLE OF CONTENTS

CHAPTER PAGE

PART II: DNA GYRASE IN *PSEUDOMONAS AERUGINOSA* STRAIN PAO

LIST OF TABLES

LIST OF FIGURES

PART I

A DEOXYRIBONUCLEASE FROM *PSEUDOMONAS AERUGINOSA* ACTIVE IN THE PRESENCE OF EDTA

CHAPTER I

INTRODUCTION

In 1976, Miller and Clark (29) began a survey of the major deoxyribonucleases of Pseudomonas aeruginosa. This study was begun in an attempt to assess the similarities or dissimilarities between the mechanisms of genetic recombination found in P. aeruginosa and Escherichia coli. They assumed that enzymes which carry out similar metabolic tasks should have similar substrate specificities and modes of action. While not all the enzymes active in the pathways leading to genetic recombination have been identified, three exonucleases have been implicated as playing a role in recombination in E. coli. These enzymes are EcoExoI (20), EcoExoV (1,4,35), and EcoExoVIII (2,19). Miller and Clark (29) searched for analogues of these three E. coli enzymes in extracts of P. aeruginosa. They identified three exonucleases in cellular extracts of P. aeruginosa. Two of the nucleases were purified and characterized. PaeExoI (29) was shown to have properties similar to EcoExoI (20). Both of these ExoI-type enzymes require Mg^{++} for maximal activity and are active only on heat-denatured DNA. Neither enzyme requires adenosine triphosphate (ATP) for activity. The second exonuclease isolated from P. aeruginosa, PaeExoV (29), is similar to

EcoExoV (1,14) in its requirements for ATP and Mg^{++}, and its use of native DNA as a substrate. ExoV nucleases isolated from E. coli and P. aeruginosa do not appear to be identical in that EcoExoV has nucleolytic activity on single-stranded DNA (14), while PaeExoV does not (29).

Miller and Clark identified but did not purify an ATP-independent nuclease active on native DNA. This study describes the purification and properties of an ATP-independent nuclease from P. aeruginosa. This nuclease has several unique properties when compared to nucleases which have been identified previously.

CHAPTER II

LITERATURE REVIEW

A. INTRODUCTION

The purpose of this chapter is to review the properties of bacterial exonucleases which have been indicated to play a role in genetic recombination. It will also review the properties of the previously characterized P. aeruginosa exonucleases and point out the similarities to other bacterial exonucleases.

B. ESCHERICHIA COLI NUCLEASES

At present, three deoxyribonucleases are known to be associated with genetic recombination in E. coli. Exonuclease I (EcoExoI) (20), exonuclease V (EcoExoV) (1,4,35), and exonuclease VIII (EcoExoVIII) (2,19). Pathways of genetic recombination have been proposed to summarize the phenotypes of mutants in which these enzymes are affected (8,9). This section contains a review of the biochemical properties of these enzymes.

EcoExoV is controlled by the recB and recC loci of E. coli (1,4). recB and recC mutants are deficient in recombination (1,4). EcoExoV is composed of two non-identical polypeptide chains with a combined molecular weight of approximately 270,000. This enzyme requires ATP

and Mg^{++} to hydrolyze linear-duplex or single-stranded DNA. It exonucleolitically degrades DNA to 5'-phosphoryl terminated oligonucleotides of an average length of 4.5 (14). The enzyme can degrade single-stranded DNA endonucleolitically. This reaction requires the presence of bovine serum albumin and is stimulated seven fold by ATP.

No EcoExoV activity has been observed on closed-circular duplex DNA. Also, closed-circular duplex DNA does not compete with substrate DNAs for the enzyme (14,22). Ultraviolet or X-ray irradiation treatment does not render closed-circular DNA susceptible to EcoExoV, but a gap of approximately 5 nucleotides is susceptible to nuclease digestion (17). DNA-RNA hybrids and psoralen treated cross-linked DNA is also resistant to degradation by EcoExoV (16).

During EcoExoV digestion 20 molecules of ATP are hydrolysed for every phosphodiester bond broken (14). The EcoExoV associated ATPase requires the presence of a polydeoxyribonucleotide for ATP hydrolysis, however, ATP hydrolysis does not need to be coupled to DNA hydrolysis, since the former reaction proceeds with the nondegradeable polymers such as RNA-DNA hybrid molecules and duplex DNA with interstrand cross-links (14,16,17).

The enzyme appears to digest linear DNA by a processive mechanism in which the rate is limited by the initiation of digestion of a DNA molecule (17). It shows no apparent polarity of degradation, nor does it appear to

be affected by the presence or absence of terminal phosphate groups (17).

Two classes of intermediates have been detected during EcoExoV digestion of duplex DNA: (a) duplex molecules with single-stranded tails of several thousand nucleotides at the 3' and the 5' termini and (b) single-stranded fragments several hundred nucleotides in length (27). MacKay and Linn have proposed the following reaction scheme: The exonuclease unwinds the strands of the duplex DNA from one end and clips off fragments several hundred nucleotides long from one strand while remaining bound to the terminus of the remaining strand. When a single-stranded tail of up to several thousand nucleotides in length is formed in this way, the enzyme switches strands and degrades the tail from its terminus, yielding a shortened, wholly duplex molecule. The cycle is then repeated until the molecule is entirely digested. This scheme recognizes the processive nature of the degradation of duplex DNA by this enzyme and hypothesizes that the ATP hydrolysis which accompanies DNA degradation is required for the unwinding of the DNA duplex (16,27).

Lehman (24) originally described a second exonuclease of E. coli (EcoExoI) which is involved in recombination. EcoExoI requires the divalent cation Mg^{++} for activity. However, EcoExoI does not require ATP and is active only on single-stranded DNA. The enzyme degrades single-stranded DNA in a 3' to 5' direction leaving acid soluble products

of 5' mono- and dinucleotides (23,24). Kushner et al. (20) have shown that this enzyme is the product of the sbcB gene. The loss of EcoExoI in recB and recC mutants increases recombinational efficiency. Therefore, the loss of EcoExoI indirectly suppresses recB and recC mutants by activation or revealing an alternate pathway of recombination (20).

Kushner et al. (19) isolated exonuclease VIII (Exo ExoVIII) from recB and/or recC mutants of E. coli which were indirectly suppressed by the presence of a mutation in the sbcA gene. They discovered that sbcA strains contained an increased level of this ATP-independent nuclease which was active on linear duplex DNA and required Mg^{++} for activity (19). Genetic and enzymatic tests indicate that this activity is not one of the previously described exonucleases in E. coli (19). Analysis on sodium dodecyl sulfate polyacrylamide gels indicated that EcoExoVIII is not present in unsuppressed ($sbcA^{+}$) strains (19). The enzyme has an approximate molecular weight of 1.4×10^{4} (13).

C. HAEMOPHILUS INFLUENZAE NUCLEASE

Friedman and Smith isolated an ATP-dependent exonuclease from Haemophilus influenzae (11). They were interested in a recombination enzyme with properties similar to EcoExoV. This enzyme requires ATP and Mg^{++} for activity (11). The enzyme digests both duplex and

single-stranded DNA (11). The enzyme attacks linear duplex DNA to give initial products which are several hundred base pairs in length (11,12). The average chain length of the limit product is 5.9 (11). The purified enzyme has a molecular weight of approximately 270,000 (11). The enzyme also has a DNA-dependent ATPase activity (39). Approximately 40 molecules of ATP are utilized for each phosphodiester bond cleaved (39). These properties of this exonuclease are very similar to EcoExoV, the recombination enzyme of E. coli.

However, unlike E. coli ATP-dependent nuclease (Eco ExoV), Wilcox and Smith found that strains lacking the H. influenzae ATP-dependent nuclease were recombinational proficient (42). These strains were assayed by transformation of competent cells with purified DNA. Results of these assays did indicate that transformation efficiency was only reduced approximately fourfold and sensitivity to radiation was slightly increased (42).

D. DIPLOCOCCUS PNEUMONIAE NUCLEASE

Vovis and Buttin explored Diplococcus pneumoniae for an ATP-dependent exonuclease to see if it was involved in transformation and recombination (40). They found an ATP-dependent nuclease which works under alkaline conditions (optimum pH from 9.25 through at least 9.8) and requires Mg^{++}. ATP was hydrolyzed during the course of DNA

degradation (39). The enzyme is much more active on native DNA than heat-denatured DNA and exhibits no activity on rRNA (40).

Vovis and Buttin examined gamma irradiation sensitive strains for recombination deficiency (41). One such strain was found to lack the ATP-dependent nuclease activity. This strain also exhibited increased sensitivity to ultraviolet irradiation (41). Genetic evidence shows that all four phenotypes are transferred as a single marker in transformation assays (41). Therefore, the loss of the ATP-dependent nuclease is responsible for increased radiation sensitivity and decreased recombination proficiency in D. pneumoniae (41).

E. BACILLUS SUBTILIS NUCLEASE

Ohi and Sueoka isolated an ATP-dependent nuclease from Bacillus subtilis (34). This enzyme digests duplex and single-stranded DNA (34). However, the nuclease only requires ATP to digest duplex DNA and not single-stranded DNA (34). Between 9 and 40 molecules of ATP are split per phosphodiester bond broken during hydrolysis of duplex DNA (34). The enzyme also requires Mg^{++} for its activity (34) and has a pH optimum of 9.5 (10).

Several recombination-deficient mutants of B. subtilis have been examined for levels of the ATP-dependent nuclease (10). Doly et al. (10) found two recombination

deficient mutants which also had a reduced level of the ATP-dependent nuclease activity. Therefore, it appears that this enzyme plays a role in recombination in B. subtilis.

F. PSEUDOMONAS AERUGINOSA NUCLEASES

Miller and Clark (29) surveyed the major deoxyribonucleases of Pseudomonas aeruginosa. They purified and characterized two nucleases from P. aeruginosa, PaeExoI and PaeExoV.

PaeExoI requires Mg^{++} to degrade linear heat denatured DNA to acid soluble products. This reaction has a pH optimum near pH 7.5 and is about 70% active at pH 7.0 and 80% active at pH 9.0. The Mg^{++} optimum of the enzyme is 2.5 mM. The enzyme is 40% active either at 12 mM magnesium or without any added magnesium, but is inhibited by disodium ethylenediamine tetraacetate (EDTA) (29).

PaeExoI will digest 100% of the substrate to acid soluble products. These products consist of 47% mononucleotides, 32% dinucleotides, 21% trinucleotides, and 2% tetranucleotides as determined by descending paper chromatography. This gives an average chain length of 1.8. The molecular weight of PaeExoI was estimated to be 6×10^4 by determining its sedimentation constant to be 4.7 in a glycerol gradient (29).

The biochemical properties of PaeExoI were interesting because they were very similar to EcoExoI which is

involved in recombination in E. coli. EcoExoI is the enzyme controlled by the sbcB gene which acts as an indirect suppressor of the RecBC phenotype in E. coli (20). Miller and Clark believed that the biochemical similarities of PaeExoI and EcoExoI may indicate that they are functionally similar. However, no recombination deficient mutants so far isolated are deficient in PaeExoI.

The second enzyme identified by Miller and Clark in P. aeruginosa was PaeExoV. PaeExoV requires ATP and Mg^{++} to digest native duplex DNA. The pH optimum of this enzyme is between 7.0 and 8.0. At pH 9.0 it is 75% active. The Mg^{++} optimum is 10 mM, and 60% maximal activity is shown at 2 and 26 mM magnesium (29). The molecular weight of PaeExoV was estimated to be 3×10^5 from its sedimentation coefficient in a glycerol gradient of 13.6 (29).

The enzyme was only active on native double-stranded DNA. There was no activity on heat-denatured DNA and no endonucleolytic activity on single-stranded DNA. Miller and Clark demonstrated greatly reduced activity on cross-linked DNA (29).

Limit digest by PaeExoV on native DNA resulted in 56% of the product being acid solubilized. Descending paper chromatography showed a distribution of products from mononucleotides to hexanucleotides among the acid soluble portion with an average chain length about 3 (29).

PaeExoV has maximal activity at 40 μM ATP. ATPase activity requires the presence of a degradable DNA species

with the exception of cross-linked DNA. When cross-linked DNA is present, ATP is hydrolyzed at approximately the same rate as when native DNA is present. Five molecules of ATP are hydrolyzed for each mole of phosphodiester bond broken (29).

PaeExoV has four properties similar to EcoExoV: (i) ATP-dependent degradation of linear native DNA; (ii) production of oligonucleotides as the small molecular weight products of digestion; (iii) the inability to degrade cross-linked native DNA; and (iv) its association with a DNA-dependent ATPase which can be activated by cross-linked native DNA (29).

PaeExoV differs from EcoExoV in that PaeExoV does not attack denatured DNA exo- or endonucleolytically as EcoExoV does (14,29). The difference in substrate is probably why PaeExoV digests only about one half of the DNA to acid soluble products while EcoExoV digests 100% of the substrate to acid soluble products (29). The similarities in PaeExoV and EcoExoV may suggest that PaeExoV plays a role in recombination in P. aeruginosa. However, PaeExoV is similar to EcoExoVIII in its ability to degrade only linear, native DNA (19,29). It is dissimilar to this enzyme in its requirement for ATP (29). PaeExoV also has similarities to the recombinational nucleases in D. pneumoniae and B. subtilis, and to the nuclease from H. influenzae. These four enzymes are all ATP-dependent

nucleases which degrade linear double-stranded DNA (11,29, 34,40). However, all but PaeExoV also degrade single-stranded DNA (11,29,34,40).

At present there are no recombination deficient mutants in P. aeruginosa which have been found to be deficient in PaeExoV activity. Mutant strains deficient in PaeExoV would be helpful in determining if PaeExoV plays a role in recombination in P. aeruginosa. Table 1.1 shows a summary of the properties of the nucleases known to be involved in recombination compared to the nucleases found in P. aeruginosa.

TABLE 1.1

PROPERTIES OF NUCLEASES FROM DIFFERENT BACTERIA COMPARED TO TWO NUCLEASES FROM *P. AERUGINOSA*

Nuclease	Gene	Active on Denatured DNA	Active on Native DNA	ATP Required
*Eco*ExoI	*sbcB*	yes	no	no
*Eco*ExoV	*recB,C*	yes	yes	yes
*Eco*ExoVIII	*sbcA*	no	yes	no
H. influenzae nuclease	--	yes	yes	yes
D. pneumoniae nuclease	--	yes[a]	yes	yes
B. subtilis nuclease	*recE*	yes[b]	yes	yes
*Pae*ExoI	--	yes	no	no
*Pae*ExoV	--	no	yes	yes

[a]Activity on denatured DNA is much less than on native DNA.

[b]ATP is not required for activity on denatured DNA.

CHAPTER III

MATERIALS AND METHODS

A. BACTERIAL STRAINS

P. aeruginosa strains used in this study were JC9006 [*pur-600*; (29)], RM40 [*pur-6000*, *trp-6*, *met-28*, *lys-56*, *str-901*; (30)] and PAO303 [*argB18*; *ch1-2*; (31)]. They are all derivatives of strain PAO (11). A thymine-requiring strain of *E. coli* B (RM1003) was used for preparation of DNA. DNA was also extracted from the *Pseudomonas* phage F116 (31).

B. PREPARATION OF DNA

Radioactively-labeled *P. aeruginosa* and *E. coli* DNAs were prepared by the method of Lehman (10) or by the method of Marmur (32) except that labeling of *P. aeruginosa* DNA was performed as described by Pemberton and Clark (36). Labeling of phage F116 DNA was carried out as described by Miller, Pemberton, and Richards (31) and purified as described by Hinkle and Miller (15). ϕX174 and λDNA was purchased from New England Biolabs. Single-stranded bacterial DNA was prepared by heating native DNA in a boiling water bath for 10 min in a solution of 0.14 *M* NaCl and 0.01 *M* sodium citrate at a concentration of 50 μg of DNA per milliliter of solution. The heat-denatured DNA was

then quickly cooled in an ice water-sodium chloride bath until the temperature had fallen below -10°C.

C. ENZYMES AND OTHER MATERIALS

Lysozyme, catalase, aldolase, Micrococcus luteus DNA polymerase, bovine serum albumin (BSA), streptomycin sulfate, thyroglobulin, sarkosyl, and trizma base were obtained from Sigma Chemical Company. T4 polynucleotide kinase was purchased from P-L Biochemicals and bacterial alkaline phosphatase was from Worthington Biochemical Corp. [^{3}H]-adenine, [^{3}H]-thymine, [γ^{32}P]-ATP, and [^{14}C]-dGTP were purchased from Amersham. Diethylaminoethylcellulose (DEAE-cellulose) was Cellex D from Bio-Rad Laboratories. Sepharose 6B was from Pharmacia Fine Chemicals, Inc. Yeast nucleic acid was from Calbiochem. Molecular weight standards for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories.

D. EXONUCLEASE ASSAY

Exonuclease activity on native or heat-denatured DNA was measured by the release of acid-soluble fragments from DNA. The standard reaction mix (0.5 ml) included potassium phosphate buffer (30 mM, pH 7.0), 2-mercaptoethanol (10 mM), and 6.6 nmol of [^{3}H]-DNA (approximately 6-8 x 10^{3} cpm/nmol from either P. aeruginosa or E. coli). Usually disodium ethylenediaminetetraacetate (EDTA) was

added to the standard reaction mixture at a final concentration of 10 mM. Reactions were incubated for 30 min at 37°C. Reactions were then terminated by placing them on ice and adding 0.2 ml of a solution of yeast nucleic acid (2 mg/ml) dissolved in 50 mM tris (hydroxymethyl) aminomethane hydrochloride (tris-HCL; pH 8.0), followed by 0.7 ml of 10% cold tricholoracetic acid. This mixture was allowed to sit on ice for 5 min. The acid-precipitable material was sedimented by centrifugation for 15 min at 3,000 rpm in a Sorval GSL-R3 centrifuge with a type HL-4 rotor. A sample (0.2 ml) of the supernatant fluid was counted in a Searle liquid scintillation counter. One unit of enzyme converts 1 nmol of native DNA to acid-soluble products in 30 min at 37°C.

E. ENDONUCLEASE ASSAY

Endonuclease activity was measured in a 0.1 ml reaction mixture containing potassium phosphate buffer (30 mM; pH 7.0), 2-mercaptoethanol (10 mM), and 1 µg of ϕX174 closed-circular virion or RF1 DNA (in separate assays). The sample to be tested for enzyme activity was added to start the reaction which was allowed to proceed at 37°C for 30 min. The reaction was stopped by adding a 10 µl portion of 5% sarkosyl, 25% glycerol, and 0.025% bromphenol blue. The samples were loaded on an 0.8% agarose gel and electrophoresed at 65 volts for 16 h (15).

They were then stained with a solution of 0.5 μg ethidium bromide/ml of H_2O for 30 min, destained in H_2O for 30 min and photographed with a Polaroid MP-4 camera using Polaroid type 57 film and a UV filter (15). Enzymatic activity was assayed as the relaxation of the closed-circular ϕX174 DNA.

F. GLYCEROL GRADIENT

Linear glycerol gradients (20 to 40%) prepared in 30 mM potassium phosphate buffer (pH 7.0) were centrifuged in a Beckman SW60 swinging-bucket rotor at 50,000 rpm (260,000xg average) for 12 h in a Beckman L5-75 ultra-centrifuge.

G. OTHER METHODS

Radioactivity was determined by the addition of 4.5 ml of scintillation fluid [18.2 g of 2,5 diphenyloxazale, 1.22 g of 1,4-bis-[2-(5-phenyloroyalel)] benzene, 2,500 ml of Triton X-100 and 4,280 ml of toluene] to 0.2 ml of aqueous-phase sample in a Searle Isocap/300 liquid scintillation counter. Protein determinations were made by the method of Lowry et al. (26), using BSA as a standard.

H. PREPARATION OF CELL EXTRACTS

Method 1 (Survey Method)

Strains were inoculated and grown in 25 ml of Luria complete broth (LB; ref. 30) in a shaking water bath (37°)

until stationary phase had been reached. Cells were harvested by centrifugation (11,000xg) and resuspension in 2.5 ml of 0.85% NaCl. The cells were repelleted and suspended in 1 ml of 10% sucrose prepared in 50 mM Tris-HCl (pH 8.0). The cells were then frozen in dry ice and ethanol and thawed in a 25°C water bath. One hundred microliters of a solution of lysozyme (2 mg/ml) dissolved in water was added to the suspension. The mixture was allowed to incubate at 0°C on ice for 60 min. Cleared lysates were prepared by centrifugation at 35,000xg for 30 min and decantation of the supernatant fluid.

Method 2 (Preparative Method)

Strain RM40 was used to inoculate large stainless steel pans (total surface area: 3.8×10^3 cm^2) containing 3 liters of Luria agar (29). These pans were allowed to incubate overnight at 37°. Cells were removed by washing the surface of the pans with LB, centrifuging the cells at 8,000xg for 15 min, resuspending the cells in 100 ml of 0.85% NaCl, and reharvesting them by centrifugation (wet weight of cells 16-20 g). Cells were suspended at a concentration of 0.5 g/ml of a 10% sucrose solution prepared in 50 mM Tris-HCl (pH 8.0). The suspension was frozen in a dry ice-ethanol bath and thawed in a 25°C water bath. The volume was measured and 0.1 ml of a solution of lysozyme (2 mg/ml, prepared in water) was added for each milliliter of cell suspension. The mixture was

allowed to incubate at 0°C for 60 min. At this point, the solution became quite viscous due to release of DNA from lysed cells. All subsequent operations in the purification of the enzyme were carried out at 4°C. Cell debris and DNA were removed by centrifugation for 30 min at 35,000xg. The supernatant fluid was decanted (fraction I).

I. ENZYME PURIFICATION

Streptomycin Sulfate Precipitation

Cell supernatant fluid prepared by cell lysis method 2 (fraction I) was treated with a 5% (wt/vol) solution of streptomycin sulfate prepared in water. The streptomycin solution was added in a proportion of 1 ml/425 optical density units at 260 nm. The mixture was stirred for 20 min, and the precipitate was separated by centrifugation for 20 min at 27,000xg. The precipitate was discarded and the supernatant fluid was dialyzed against 2 changes of 1.5 liters each of 50 mM Tris-HCl buffer (pH 8.0), 0.1 mM dithiothreitol, 0.1 mM EDTA, and 10% (wt/vol) glycerol for 6 h to produce fraction II.

First DEAE-Cellulose Chromatography

A DEAE-cellulose column (2.5 x 12.5 cm) was equilibrated with 50 mM Tris-HCl (pH 8.0), 0.1 mM dithiothreitol, 0.1 mM EDTA, and 30% glycerol (buffer A). Fraction II was made 30% (wt/vol) glycerol and was layered directly onto the column, followed by a 35 ml wash of

buffer A. A 300 ml gradient of 0.05 to 0.3 M NaCl in buffer A followed. A flow rate of 40 ml/h was maintained. Nuclease activity on native DNA eluted as a single broad peak from 0.1 to 0.2 M NaCl. Active fractions were pooled and concentrated in an Amicon Pressure Concentrator to 7 ml. The concentrate was dialyzed against 2 changes of 1 liter each of 0.03 M potassium phosphate buffer (pH 7.0), 0.1 mM dithiothreitol, 0.1 mM EDTA, and 30% (wt/vol) glycerol (buffer B) for 6 h to make fraction III.

Sepharose 6B Chromatography

Fraction III was layered onto a column of Sepharose 6B which had been equilibrated with buffer B. The column dimensions were 1.6 x 86 cm. The column was eluted with buffer B and fractions (25 drops) were collected. The nuclease activity eluted about five fractions after blue dextran. Active fractions were pooled to make fraction IV.

Second DEAE-Cellulose Chromatography

A second DEAE-cellulose column (0.8 x 7.5 cm) was equilibrated with buffer B. Fraction IV (12 ml) was layered directly onto the column, followed by a 3 ml wash of buffer B. A 50 ml gradient of 0.0 to 0.7 M KCl in buffer B followed. A flow rate of 30 ml/h was maintained. The nuclease activity on native DNA which eluted between 0.05 and 0.12 M KCl was pooled and concentrated with a Millipore Submersible Concentrator to make fraction V.

Glycerol Gradient

Fraction V was layered onto a preparative 20-40% (wt/vol) linear glycerol gradient and centrifuged in a Beckman SW 41 swinging-bucket rotor at 40,000 rpm (193,000 xg average) for 16 h in a Beckman L5-75 ultracentrifuge. Gradients were fractionated and active fractions were pooled to make fraction VI.

J. POLYACRYLAMIDE GEL ELECTROPHORESIS

Nondenaturing polyacrylamide gel electrophoresis was carried out as described by Clanton et al. (7) and SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (21) except that 7.5% (wt/vol) acrylamide gels were used in all cases.

K. PREPARATION OF 3' AND 5' END LABELED LAMBDA DNA

Labeling of the 5' Terminus with ^{32}P

Unlabeled λ DNA was treated with bacterial alkaline phosphatase to remove the 5' phosphate groups. The reaction mixture was extracted twice with phenol and dialyzed as described by Richardson (38). The dialyzed solution contained no detectable phosphatase activity. The DNA was incubated with T4 polynucleotide kinase and [$\gamma^{32}P$]-ATP by the procedure described by Richardson (38). The product of this reaction was λ DNA labeled on the 5' end with ^{32}P.

Labeling of the 3' Terminus with ^{14}C

Five-prime-^{32}P labeled λ DNA was labeled with ^{14}C at the 3' end by treatment with Micrococcal DNA polymerase in the presence of dATP, TTP, dCTP, and [^{14}C]-dGTP. The reaction incorporated [^{14}C]-dGTP as the radioactive label in the 3' end of λ DNA. The procedure according to Bollum was followed (3). This results in λ DNA with a 5'-^{32}P label and a 3'-^{14}C label.

CHAPTER IV

RESULTS

A. DETECTION OF NUCLEASE ACTIVITY IN CELL LYSATES

Cell extracts of RM40 were prepared by Method 1 and surveyed to detect nuclease activity on native DNA (Table 1.2). In the absence of added Mg^{++}, the level of ATP-independent nuclease activity was stimulated two- to three-fold over the activity detected in the presence of added Mg^{++}. The activity was resistant to inhibition by the addition of 10 mM EDTA to the reaction mixture and may have been slightly stimulated.

Cleared lysates were prepared by Method 1 from two additional strains of P. aeruginosa (JC9006 and PAO303) and were assayed for nuclease activity on native DNA in the presence of EDTA. Each was found to exhibit EDTA-resistant nuclease activity. These findings encouraged attempts to purify this nuclease activity.

B. PURIFICATION OF THE EDTA-RESISTANT NUCLEASE

To begin purification, cell extracts of P. aeruginosa strain RM40 were prepared by Method 2 as described above. The crude cell extracts were subjected to a variety of fractionation procedures (Table 1.3) and purification was followed by assaying the degradation of native DNA in the

TABLE 1.2

NUCLEASE ACTIVITY IN CELL LYSATES OF RM40 UNDER VARIOUS CONDITIONS[a]

Additions to Standard Reaction Mixture	Units/mg Protein
None	13.5
$MgSO_4$ (10 mM)	5.6
$MgSO_4$ (10 mM) and ATP (40 μM)	8.7
ATP (40 μM)	14.2
EDTA (10 mM)	15.0

[a]Native DNA isolated from E. coli was used as the substrate.

TABLE 1.3

PURIFICATION OF THE NUCLEASE[a]

Fraction	Description	Volume (ml)	Total Protein (mg)	Specific Activity (Units/mg Protein)	Recovery (%)
I	Cell supernatant fluid	50	205	18	100
II	Streptomycin SO_4 supernatant fluid	49	132	25	86
III	DEAE-Cellulose I	7	32	90	75
IV	Sepharose 6B	12	6	401	59
V	DEAE-Cellulose II	4	1.0	782	20
VI	Glycerol gradient	2	0.047	1281	3

[a]All reactions were carried out in the presence of 10 mM EDTA in the standard reaction mixture using native E. coli DNA as the substrate.

presence of EDTA. This procedure gave a 71-fold purification with 3% recovery.

C. PROPERTIES OF THE NUCLEASE IN FRACTION V

The activity of the purified enzyme was increased by one-third when EDTA (10 mM) was added to the reaction mixture. When Mg^{++} or several other divalent cations were added to the reaction mixture the enzymatic activity was reduced (Table 1.4). Ferrous ion inhibited the activity completely. Because of these effects, 10 mM EDTA was added to all subsequent assays reported in this study.

The nuclease has a pH optimum between pH 8.0 and 9.0 and retains greater than 80% of its maximal activity in the pH range of 6.5 to 9.5. At pH 5.0, however, the enzyme has only 40% of its maximum activity, whereas at pH 10.5, it is 70% as active (Figure 1.1).

The nuclease is equally active on native DNA isolated from P. aeruginosa, E. coli, and the Pseudomonas phage F116. One hundred percent of the native-DNA substrate is rendered acid-soluble in reactions allowed to proceed to completion. The enzyme degrades heat-denatured, linear DNA with the same efficiency as native DNA. To insure that these multiple activities are properties of the same protein molecule, a sample of the enzyme preparation was sedimented through a 20 to 40% glycerol gradient and fractions were collected. Each fraction was assayed on

TABLE 1.4

EFFECTS OF DIVALENT CATIONS ON NUCLEASE ACTIVITY

Reaction Mixtures[a]	Relative Activity (Percent)
30 mM Tris-maleate-NaOH (pH 7.0)	100
+ 5 mM $MgCl_2$	80
+ 5 mM $MnCl_2$	50
+ 5 mM $ZnSO_4$	80
+ 5 mM $FeSO_4$	0
+ 5 mM $CaCl_2$	87

[a]Tris-maleate-NaOH buffer was substituted for potassium phosphate buffer because Mn^{++} and Ca^{++} form precipitates in the presence of potassium phosphate. The reaction mixture also contained 6.6 nmol of native DNA isolated from E. coli and 10 mM 2-mercaptoethanol.

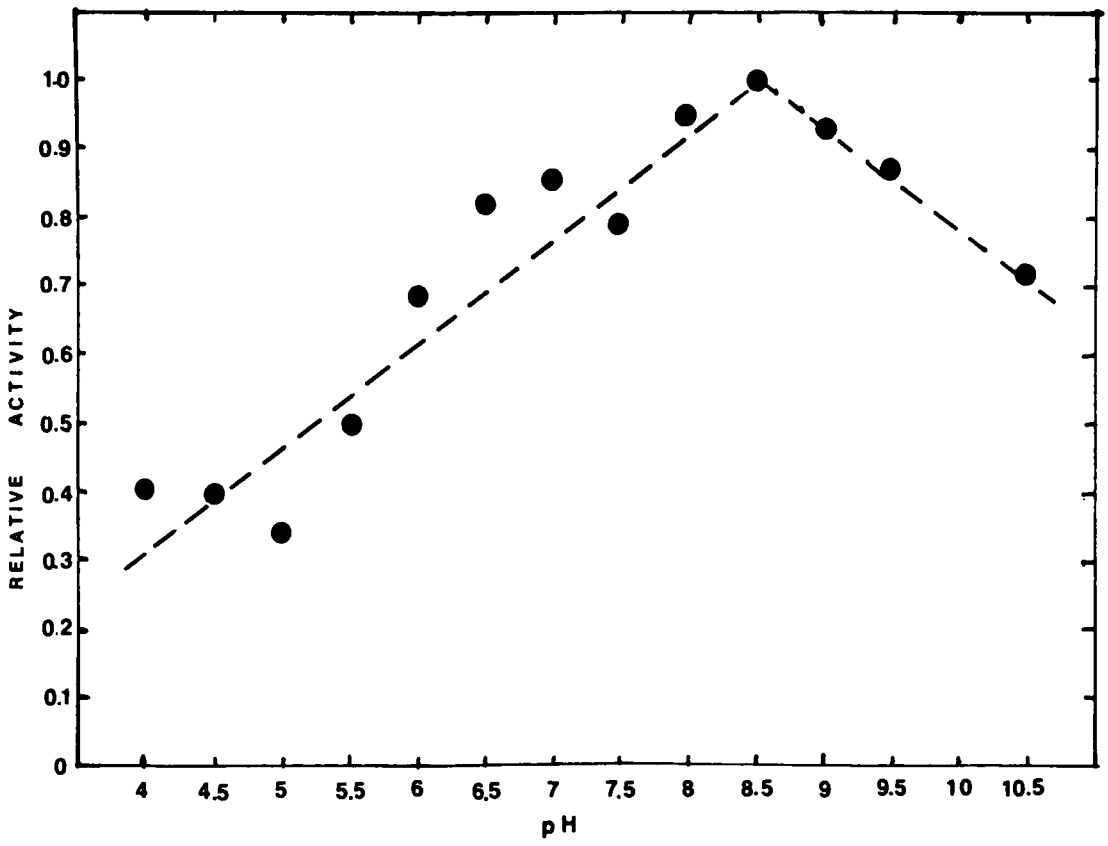

Figure 1.1. Relative activity of the nuclease at various pHs on native DNA.

both native and heat-denatured DNA in the presence of EDTA (Figure 1.2). A common peak of activity was observed. Fractions demonstrating similar enzymatic activity were then pooled from preparative glycerol gradients and electrophoresed on nondenaturing polyacrylamide gels. This pool contained only one discernible protein species.

Fraction V was tested for endonuclease activity as described in Materials and Methods. No enzymatic activity could be demonstrated when covalently-closed-circular ϕX174 RF1 DNA (double-stranded) or when closed-circular ϕX174 virion DNA (single-stranded) was used as a substrate in reaction mixtures containing 10 mM EDTA.

D. MOLECULAR WEIGHT DETERMINATION

The molecular weight of the enzyme in fraction V was estimated to be $1.6 \pm 0.1 \times 10^5$ by determining its sedimentation coefficient in a linear 20 to 40% (wt/vol) glycerol gradient (Figure 1.2). This molecular weight was calculated from the sedimentation coefficient (8S), using the method of Martin and Ames (28).

Fraction VI yielded a single band on nondenaturing polyacrylamide gels. Fraction VI was applied to a sodium dodecyl sulfate polyacrylamide gel and stained after electrophoresis as described in Materials and Methods. Two protein species were revealed: one with a molecular weight of 69,000 and the other with a molecular weight of 78,000 (Figure 1.3).

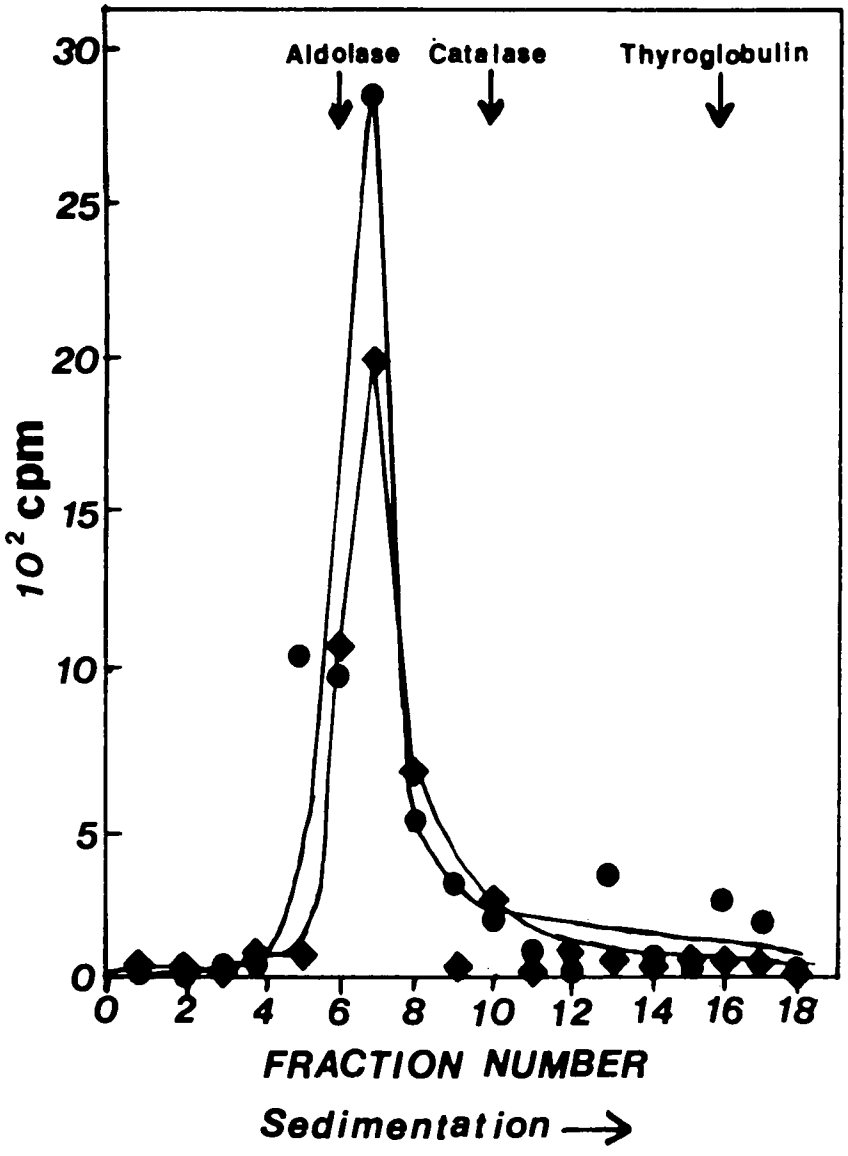

Figure 1.2. Sedimentation of the EDTA-resistant nuclease from fraction V in glycerol gradients (20 to 40%). The gradients were centrifuged in a Beckman SW60 rotor at 260,000xg as described in the text. Each fraction was assayed in the standard reaction mixture to which 10 mM EDTA and either native (●) or heat-denatured (◆) E. coli [^{3}H]-DNA had been added. Aldolase (7.35$S_{20,w}$; MW: 149,000), catalase (11.3$S_{20,w}$; MW: 247,000), and thyroglobulin (19.3$S_{20,w}$; MW: 669,000) were sedimented in parallel gradients and used as sedimentation standards.

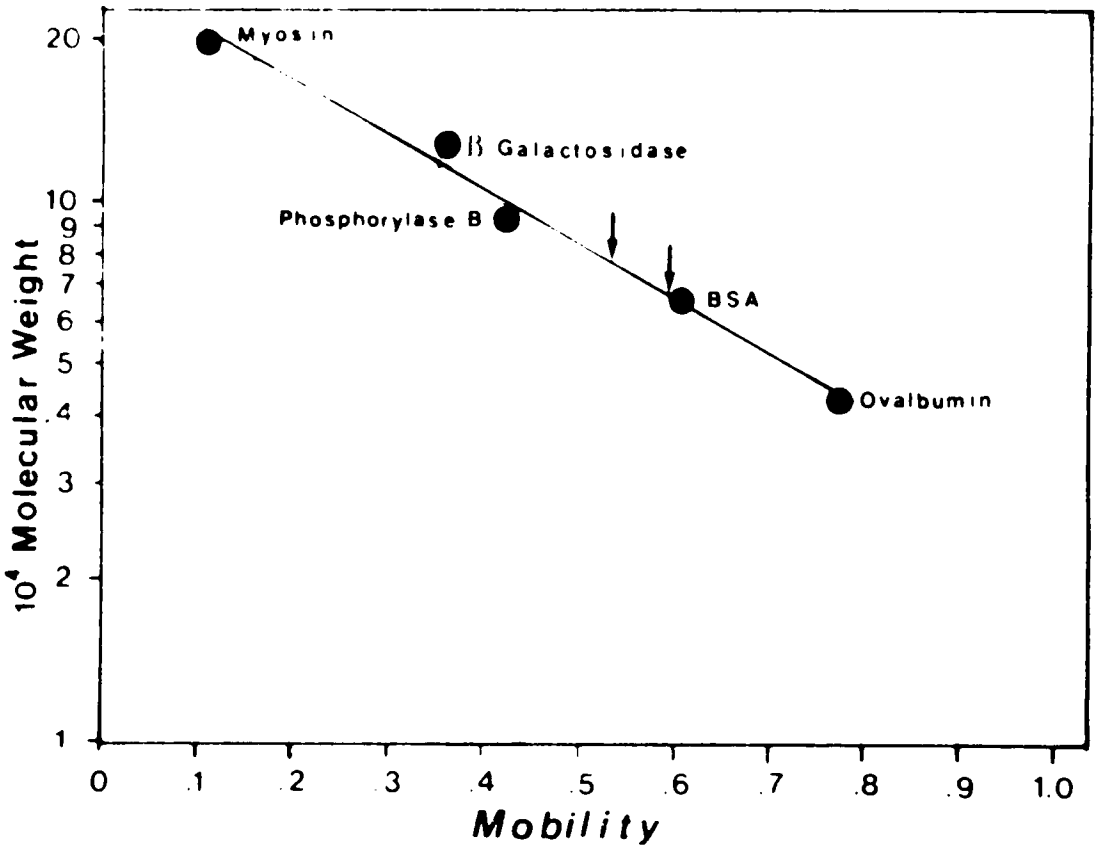

Figure 1.3. Molecular weight determinations of the denatured EDTA-resistant nuclease from fraction VI by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrophoresis was carried out as described in the text using myosin (MW: 200,000), β-galactosidase (MW: 116,000), phosphorylase B (MW: 94,000), BSA (MW: 68,000), and ovalbumin (MW: 43,000) as standards. Two Coomassie Brilliant Blue-staining species were detected as indicated by arrows.

E. DIRECTION OF DEGRADATION BY THE *P. AERUGINOSA* NUCLEASE

To determine if the *P. aeruginosa* nuclease initiates hydrolysis at the 3' termini or the 5' termini of DNA strands, DNA labeled with ^{32}P at the 5'-end and ^{14}C at the 3'-end were incubated for various times with the nuclease, and the amount of acid-soluble radioactivity was determined. The preliminary experiments shown in Figure 1.4 indicate that both the ^{32}P label and the ^{14}C label are rapidly degraded. This is true for both native DNA and heat-denatured DNA (Figure 1.4).

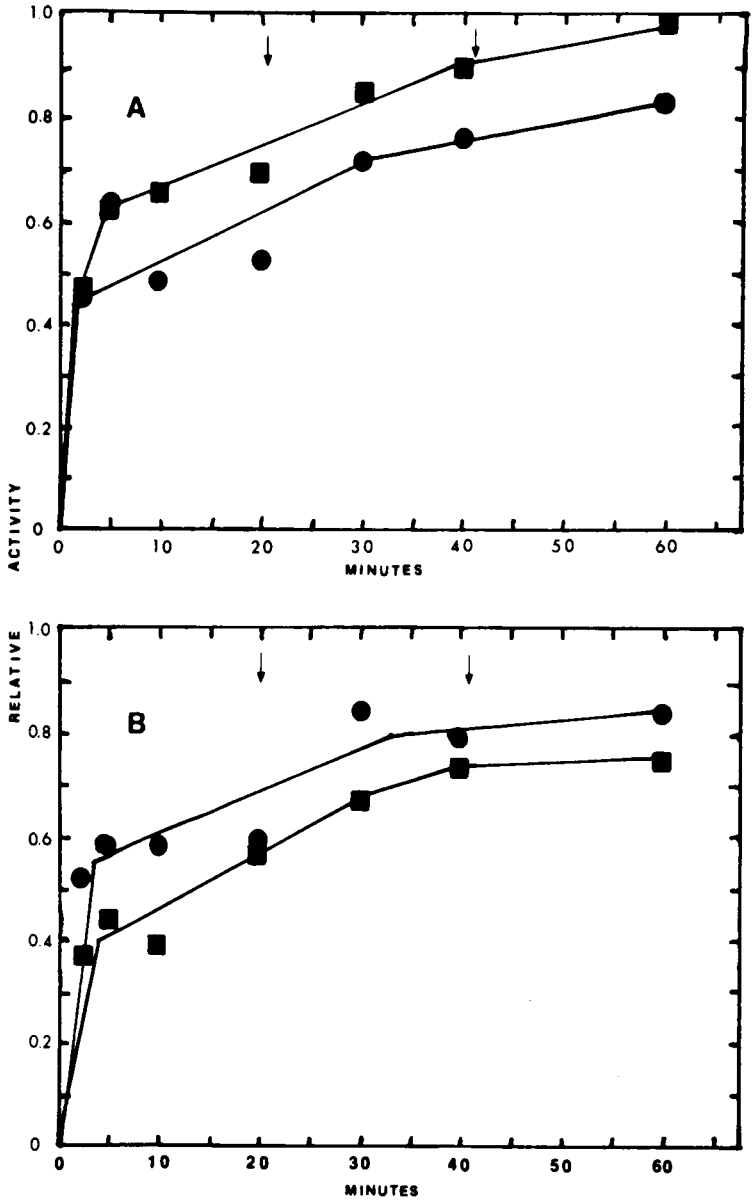

Figure 1.4. Relative rates of release of terminal label from λ DNA. DNA was prepared as described in Materials and Methods. A. Native λ [^{32}P]-5' and [^{14}C]-3' DNA. B. Heat-denatured λ [^{32}P]-5' and [^{14}C]-3' DNA. Samples were assayed at various times as described in Materials and Methods. (●) ^{32}P; (■) ^{14}C label.

CHAPTER V

DISCUSSION

An EDTA-resistant deoxyribonuclease has been purified to electrophoretic homogeneity from P. aeruginosa strain RM40. This enzyme is equally active in the hydrolysis of native DNA and heat-denatured DNA. The enzyme cannot hydrolyze circular, single-stranded DNA or circular double-stranded DNA. Because a DNA molecule with a free terminus is required to initiate hydrolysis, this enzyme was classified as an exonuclease and has been named exonuclease IX (PaeExoIX) in accordance with the nomenclature proposed by Miller and Clark (29). This enzyme is different from other exonuclease activities described in P. aeruginosa (29) or E. coli (6).

PaeExoIX is most active when an agent capable of chelating divalent cations is added to the reaction mixture. This enzyme is unique among the exonucleases isolated from E. coli (6) and P. aeruginosa (29). Of this group, only EcoExoVII shows any activity (albeit reduced) in the presence of EDTA (6). EcoExoVII is active only on heat-denatured DNA and is stimulated by Mg^{++} (6).

The data reported here indicate that PaeExoIX has a molecular weight of approximately 160,000 and is composed of two nonidentical polypeptides of slightly different

sizes. The larger polypeptide has a mass of 78,000 daltons while the mass of the smaller subunit is 69,000 daltons.

The data on the direction of degradation indicate that both the 3'-end and the 5'-end are attacked by the nuclease equally, or that the reaction is processive and very fast. Karu et al. (16) have shown that EcoExoV attacked both the 3'-end and the 5'-end of single-stranded and double-stranded DNA. These data for the P. aeruginosa nuclease are very similar to the EcoExoV data for direction of degradation.

PaeExoIX has some properties in common with EcoExoV in that it is active on native as well as heat-denatured DNA. However, it differs from EcoExoV in that it does not require Mg^{++} or ATP for activity and has no endonucleolytic activity. The ability of PaeExoIX to degrade both native and heat-denatured DNA exonucleolytically suggest the possibility that this enzyme may be a functional analogue of EcoExoV in the processes of recombination. Exonucleases which are active in degrading both native and heat-denatured DNA have been shown to have a function in the recombination of genetic material in several species of bacteria including E. coli (1,18,35,43), H. influenzae (12,22,42), D. pneumoniae (40,41), and B. subtilis (10). While each of these enzymes also hydrolyzes ATP as an integral part of the hydrolysis of DNA, the function of this ATP hydrolysis is not clear. ATP hydrolysis can be uncoupled from the

exonuclease activity of EcoExoV by presenting the enzyme with a nondegradeable substrate such as cross-linked DNA (16). The process of recombination may not require that the hydrolysis of both ATP and DNA be a function of the same protein molecule. In fact, the ATP-independent exonuclease EcoExoVIII appears to substitute for the activities of EcoExoV associated with recombination in E. coli strains carrying recB or recC and sbcA mutations (2,19) and LamExoI, the enzyme responsible for generalized recombination in coliphage lambda, is an ATP-independent exonuclease (5,25). PaeExoIX is also similar to EcoExoI in that it does not require ATP for the degradation of single-stranded DNA (24).

Table 1.5 summarizes the properties of the known bacterial recombinational nucleases and compares them to the properties of exonuclease IX from P. aeruginosa. While none are identical to PaeExoIX, there are some similarities to the recombinational nucleases in these other bacteria.

The metabolic role of PaeExoIX is not known. PaeExoIX may be involved in recombination or irradiation sensitivity since it has similar biochemical properties to several nucleases which are involved in these metabolic processes. The best way to test the metabolic role of this enzyme would be to have mutant strains of P. aeruginosa deficient in PaeExoIX. This could be done by two methods.

TABLE 1.5

PROPERTIES OF NUCLEASES FROM SELECTED BACTERIA COMPARED TO EXONUCLEASE IX FROM *P. AERUGINOSA*

Nuclease	Gene	Active on Denatured DNA	Active on Native DNA	ATP Required
EcoExoI	*sbcB*	yes	no	no
EcoExoV	*recB,C*	yes	yes	yes
EcoExoVIII	*sbcA*	no	yes	no
H. influenzae nuclease	--	yes	yes	yes
D. pneumoniae nuclease	--	yes[a]	yes	yes
B. subtilis nuclease	*recE*	yes[b]	yes	yes
PaeExoIX	--	yes	yes	no

[a]Activity on denatured DNA is much less than on native DNA.

[b]ATP is not required for activity on denatured DNA.

One method would be to obtain a large number of irradiation sensitive and recombination deficient mutants of P. aeruginosa and screen these for levels of PaeExoIX activity. A deficiency of PaeExoIX in one of these mutants would indicate a possible role for this enzyme in one of these metabolic processes. A second method to determine the metabolic role of PaeExoIX would be to mutagenize cells of P. aeruginosa and examine the survivors for a deficiency in PaeExoIX. Once a mutant is found in this way, its phenotypic characteristics can be tested to determine if there is a reduced recombinational efficiency or an increased irradiation sensitivity. These experiments would indicate if PaeExoIX is involved in these metabolic processes.

LIST OF REFERENCES

LIST OF REFERENCES

1. Barbour, S. B. and A. J. Clark. 1970. Biochemical and genetic studies on recombination proficiency in Escherichia coli. I. Enzymatic activity associated with $recB^+$ and $recC^+$ genes. Proc. Natl. Acad. Sci. U.S.A. 65:955-961.

2. Barbour, S. D., H. Nagaishi, A. Templin, and A. J. Clark. 1970. Biochemical and genetic studies on recombination proficiency in Escherichia coli. II. Rec^+ revertants caused by indirect suppression of Rec^- mutants. Proc. Natl. Acad. Sci. U.S.A. 67: 128-135.

3. Bollum, F. J. 1966. Preparation of deoxyribonucleotide polymerizing enzyme from calf thymus gland, pp. 284-286. In G. L. Cantoni and D. R. Davies (ed.), Procedures in Nucleic Acid Research. Harper and Row, New York.

4. Buttin, G. and M. R. Wright. 1968. Enzymatic DNA degradation in Escherichia coli: its relationship to synthetic processes at the chromosome level. Cold Spring Harbor Symp. Quant. Biol. 33:259-269.

5. Carter, D. M. and C. M. Radding. 1971. The role of exonuclease and β protein of phage λ in genetic recombination. J. Biol. Chem. 246:2502-2512.

6. Chase, J. W. and C. C. Richardson. 1974. Exonuclease VII of Escherichia coli: mechanism of action. J. Biol. Chem. 249:4553-4561.

7. Clanton, D. J., W. S. Riggsby, and R. V. Miller. 1979. NgoII, a restriction endonuclease from Neisseria gonorrhoeae. J. Bacteriol. 137:1299-1307.

8. Clark, A. J. 1973. Recombination deficient mutants of E. coli and other bacteria. Annu. Rev. Genet. 7:67-86.

9. Clark, A. J. 1974. Progress toward a metabolic interpretation of genetic recombination in Escherichia coli and bacteriophage lambda. Genetics 78:259-271.

10. Doly, J., E. Sasarman, and C. Anagnostopoilos. 1974. ATP-dependent deoxyribonuclease in Bacillus subtilis and a mutant deficient in this activity. Mutat. Res. 22:15-23.

11. Friedman, E. A. and H. O. Smith. 1972a. An adenosine triphosphate-dependent deoxyribonuclease from Hemophilus influenzae Rd. I. Purification and properties of the enzyme. J. Biol. Chem. 247:2846-2853.

12. Friedman, E. A. and H. O. Smith. 1972b. An adenosine triphosphate-dependent deoxyribonuclease from Hemophilus influenzae Rd. III. Substrate specificity. J. Biol. Chem. 247:2859-2865.

13. Gillen, J. R., A. E. Karu, H. Nagaishi, and A. J. Clark. 1977. Characterization of the deoxyribonuclease determined by lambda reverse of exonuclease VIII of Escherichia coli. J. Mol. Biol. 113:27-41.

14. Goldmark, P. J. and S. Linn. 1972. Purification and properties of the recBC DNase of Escherichia coli K-12. J. Biol. Chem. 247:1849-1860.

15. Hinkle, N. F. and R. V. Miller. 1979. pMG-7 mediated restriction of Pseudomonas aeruginosa phage DNAs is determined by a class II restriction endonuclease. Plasmid 2:387-393.

16. Karu, A. F. and S. Linn. 1972. Uncoupling of the recBC ATPase from DNase by DNA crosslinked with psoralen. Proc. Natl. Acad. Sci. U.S.A. 69:2855-2859.

17. Karu, A. E., V. MacKay, P. J. Goldmark, and S. Linn. 1973. The recBC deoxyribonuclease of Escherichia coli K-12. J. Biol. Chem. 248:4874-4881.

18. Kushner, S. R. 1974. Differential thermolability of exonuclease and endonuclease activities of recBC nuclease isolated from the thermosensitive recB and recC mutants. J. Bacteriol. 120:1219-1222.

19. Kushner, S. R., H. Nagaishi, and A. J. Clark. 1974. Isolation of exonuclease VIII: the enzyme associated with the sbcA indirect suppressor. Proc. Natl. Acad. Sci. U.S.A. 71:3593-3597.

20. Kushner, S. R., H. Nagaishi, A. Templin, and A. J. Clark. 1971. Genetic recombination in Escherichia coli: the role of esonuclease I. Proc. Natl. Acad. Sci. U.S.A. 68:824-685.

21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

22. LeClere, J. E. and J. K. Setlow. 1974. Transformation in Haemophilus influenzae, pp. 187-207. In R. R. Grell (ed.), Mechanisms in recombination. Plenum Press, New York.

23. Lehman, I. R. 1960. The deoxyribonucleases of Escherichia coli. I. Purification and properties of a phosphodiesterase. J. Biol. Chem. 235: 1479-1487.

24. Lehman, I. R. and A. L. Nussbaum. 1964. The deoxyribonuclease of Escherichia coli. V. On the specificity of exonuclease I (phosphodiesterase). J. Biol. Chem. 239:2628-2636.

25. Little, J. W., I. R. Lehman, and A. D. Kaiser. 1967. An exonuclease induced by bacteriophage λ. J. Biol. Chem. 242:672-678.

26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265-275.

27. MacKay, V. and S. Linn. 1974. The mechanism of degradation of duplex deoxyribonucleic acid by the recBC enzyme of Escherichia coli K-12. J. Biol. Chem. 249:4286-4294.

28. Martin, R. G. and B. W. Ames. 1961. A method for determining sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372-1379.

29. Miller, R. V. and A. J. Clark. 1976. Purification and properties of two deoxyribonucleases of Pseudomonas aeruginosa. J. Bacteriol. 127:794-802.

30. Miller, R. V. and C. M. C. Ku. 1978. Characterization of Pseudomonas aeruginosa mutants deficient in the establishment of lysogeny. J. Bacteriol. 134: 875-883.

31. Miller, R. V., J. M. Pemberton, and K. E. Richards. 1974. F116, D3, and G101: temperate bacteriophages of Pseudomonas aeruginosa. Virology 59: 566-569.

32. Murmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.

33. Ohi, S., D. Bastia, and N. Sueoka. 1974. Non-specific "pairing" of DNA molecules by recombination enzyme of Bacillus subtilis. Nature 248:586-588.

34. Ohi, S. and N. Sueoka. 1973. ATP-dependent deoxyribonuclease in Bacillus subtilis. Purification and properties. J. Biol. Chem. 248:7336-7344.

35. Oishi, M. 1969. An ATP-dependent deoxyribonuclease from Escherichia coli with a possible role in genetic recombination. Proc. Natl. Acad. Sci. U.S.A. 65:1292-1299.

36. Pemberton, J. M. and A. J. Clark. 1973. Detection and characterization of plasmids in Pseudomonas aeruginosa strain PAO. J. Bacteriol. 144:424-433.

37. Pemberton, J. M. and B. W. Holloway. 1972. Chromosome mapping in Pseudomonas aeruginosa. Genet. Res. 19:251-260.

38. Richardson, C. C. 1965. Phosphorylation of nucleic acid by an enzyme from T4 bacteriophage-infected Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 54:158-165.

39. Smith, H. O. and E. A. Freidman. 1972. An adenosine triphosphate-dependent deoxyribonuclease from Hemophilus influenzae Rd. II. Adenosine triphosphatase properties. J. Biol. Chem. 247:2854-2858.

40. Vovis, G. F. and G. Buttin. 1970a. An ATP-dependent deoxyribonuclease from Diplococcus pnenoniae. I. Partial purification and some biochemical properties. Biochim. Biophys. Acta. 224:29-41.

41. Vovis, G. F. and G. Buttin. 1970b. An ATP-dependent deoxyribonuclease from Diplococcus pneumoniae. II. Evidence for its involvement in bacterial recombination. Biochim. Biophys. Acta. 224:42-54.

42. Wilcox, K. W. and H. O. Smith. 1975. Isolation and characterization of mutants of Haemophilus influenzae deficient in an adenosine 5'-triphosphate-dependent deoxyribonuclease activity. J. Bacteriol. 122:443-453.

43. Wright, M., G. Buttin, and J. Hurwitz. 1971. The isolation and characterization from Escherichia coli of an adenosine triphosphate-dependent deoxyribonuclease directed by recB,C genes. J. Biol. Chem. 246:6543-6555.

PART II

DNA GYRASE IN *PSEUDOMONAS AERUGINOSA* STRAIN PAO

CHAPTER I

INTRODUCTION

DNA gyrase (12,25,41) in the presence of adenosine triphosphate (ATP), converts relaxed closed-circular DNA to a negative supercoiled form. In the absence of ATP, DNA gyrase will relax supercoiled DNA (11). Escherichia coli DNA gyrase is composed of two polypeptides one 95,000 daltons and the other 105,000 daltons in size (16). Studies by Gellert and colleagues (14) demonstrated that the structurally related drugs novobiocin and coumermycin A_1 inhibit DNA gyrase. Resistance to these drugs is controlled by the gene gyrB (formally cou) in E. coli, and DNA gyrase from strains containing a mutation in the gyrB gene is resistant to novobiocin and coumermycin A_1 in vitro (14). Reconstitution studies have shown gyrB to be the structural gene for the 95,000 dalton polypeptide of DNA gyrase (16). Additional studies have demonstrated that DNA gyrase is also the target for the drugs nalidixic acid and oxolinic acid (11,43). Resistance to these drugs is the consequence of a mutation in the gene gyrA (formally nalA) which codes for the 105,000 dalton polypeptide of DNA gyrase (11,16).

DNA gyrase has been implicated in the integration of the lambda prophage in E. coli (21). Because of my interest

in the biochemical role of the host organism in recombination and the establishment of lysogeny in Pseudomonas aeruginosa (28,29) prompted a search for DNA gyrase in this species. This study describes the identification of a DNA gyrase activity from P. aeruginosa and some of its properties.

CHAPTER II

LITERATURE REVIEW

A. EARLY OBSERVATIONS OF DNA GYRASE

Most species of duplex DNA exist naturally in a negatively supertwisted form containing about one negative supercoil per 15 double helical twist (1). The supertwisting of DNA is an important part of the process of DNA replication, transcription, and recombination (7). Gellert et al. discovered a bacterial enzyme which introduces negative supertwists into DNA, this enzyme has been named DNA gyrase (12). DNA gyrase requires ATP, Mg^{++}, and a relaxed closed-circular DNA substrate for supercoiling.

Wang initiated the enzymology of supercoiling with the discovery of E. coli ω protein (46). This enzyme required no co-factor and removes most of the negative supercoils of DNA (46). E. coli ω protein has been designated Eco DNA topoisomerase I. Topoisomerases have also been identified in many eukaryotic organisms (7).

DNA gyrase (topoisomerase II) is the only known topoisomerase that catalyses supercoiling of DNA. DNA gyrase was discovered by the observation that bacteriophage λ required a negatively supercoiled DNA to undergo integrative recombination. Nash et al. discovered that crude lysates of E. coli could support in vitro integrative

recombination with a relaxed closed-circular bacteriophage λ DNA (33). This activity was purified and named DNA gyrase (12). Gellert et al. later found that novobiocin and coumermycin A_1, two related inhibitors of nucleic acid synthesis *in vivo*, were inhibitors of DNA gyrase *in vitro* (14). The effect of these drugs is abolished by a mutation in the *cou* (now *gyrB*) gene, which controls the cellular level of sensitivity to these drugs (7,14).

Sugino et al. (43) and Gellert et al. (11) discovered that DNA gyrase was inhibited by the drug nalidixic acid and the more potent analog oxolinic acid. Both these drugs inhibit DNA replication. The target protein for these drugs is the product of the *nalA* (now *gyrA*) gene. This protein is also a component of DNA gyrase (11,16,43).

B. STRUCTURE OF DNA GYRASE

Both *E. coli* and *Micrococcus luteus* DNA gyrase have been purified to homogeneity. Each has two subunits. *E. coli* subunits are called A and B which are the target protein for nalidixic acid and novobiocin, respectively (13,34). *M. luteus* gyrase subunits α and β are functionally identical to the *E. coli* subunits A and B, respectively. Independent studies on both organisms revealed that only a small portion of the subunits of gyrase are associated in cell extracts. The subunits can be reconstituted under the appropriate conditions (7).

Subunit A consists of 105,000 daltons and subunit B consists of 95,000 daltons (16). Subunit A prepared from a nalA mutant reconstituted to subunit B from a wild type cell is resistant to nalidixic acid (16). Subunit B prepared from a cou mutant reconstituted to purified wild type subunit A is resistant to coumermycin A_1. Subunit A purified from a nalA temperature-sensitive mutant is thermolabile (22,23). Also, attachment of a radioactive ATP derivative to subunit B of gyrase is specifically inhibited by novobiocin (31). Therefore, the structural gene for subunit A is nalA and the structural gene for subunit B is cou. These genes are now called gyrA and gyrB, respectively (7).

When gyrase is incubated with duplex DNA, a salt stable complex is formed. Wang analyzed this complex by density gradient centrifugation in CsCl and found that gyrase binds to DNA as a tetrameric unit, presumably of the $\alpha_2\beta_2$ type (47). Peebles et al. (34) discovered that the native molecular weight of subunit A is 220,000. Therefore, subunit A appears to be a dimer of two identical protomers (34). Equal molar amounts of subunits A and B reconstitute maximal gyrase activity (16,43). Therefore, the best estimate for the molecular weight of the holoenzyme is 400,000, the value expected for an $\alpha_2\beta_2$ structure (7,31,47).

C. ACTIVITIES OF GYRASE

During supercoiling gyrase remains bound to its DNA substrate (7). Supercoiling is processive (7) and catalytic

(16,29), one molecule introduces about 100 supertwists per minute at 30°C. Gyrase must have a coupled energy component since supercoiling is an endergonic reaction. Gyrase hydrolyzes ATP to adenosine diphosphate (ADP) and inorganic phosphate in the presence of DNA (4,7,31,42). The ATPase activity is inhibited by novobiocin (7,13).

DNA gyrase relaxes negative supercoils in the absence of ATP (11,43) but cannot relax positive supercoils (3,25). The rate of relaxation is lower than the rate of supercoiling (16). Relaxation is inhibited by nalidixic acid but not by novobiocin (7).

When sodium dodecyl sulfate (SDS) is added to a nalidixic acid treated gyrase reaction, the DNA undergoes cleavage of both polynucleotide strands. A gyrA polypeptide is left attached to each end of the broken polypeptide strand. This implies that the activity of gyrase involves breakage and reunion of both strands. Morrison and Cozzarelli have shown this cleavage to be highly site specific (32).

The most recent observation of the DNA gyrase reaction is catenation, the interlocking of duplex DNA rings and the resolution of catenanes into component circles. Catenanes are important structures physiologically. They are found among circular DNA molecules and are sometimes the predominate species. Catenation and uncatenation provide evidence that gyrase makes a double strand break,

passes the DNA through the break, and reseals the break in an efficient reaction (7,24).

The activities of DNA gyrase are summarized in Figure 2.1 and the inhibition of these activities by novobiocin and nalidixic acid are summarized in Table 2.1. These properties are shared by DNA gyrases identified in E. coli (7,13,24), M. luteus (7,25), and Bacillus subtilis (7,41), except catenation and uncatenation which has not been tested with DNA gyrase from B. subtilis (41). Even the specific sites of cleavage are the same in the presence of oxolinic acid and SDS with all three enzymes (3,7,41).

D. MECHANISM OF SUPERCOILING BY DNA GYRASE

Work reported by Brown and Cozzarelli indicates that both the introduction and the removal of supertwist by DNA gyrase change the linking number of DNA in steps of two (2). The linking number, Lk, is the algebraic sums of the number of supercoils, Sc, and the number of double-helical turns, Tr, in a closed-circular duplex DNA:

$$Lk = Sc + Tr$$

The linking number cannot be changed without breaking one or both strands of the duplex helix. The most likely model to explain how the linking number of DNA changes in steps of two is called the sign inversion model. The sign inversion model consists of the following steps: (i) Gyrase binds to a DNA molecule stabilizing a positive supercoil

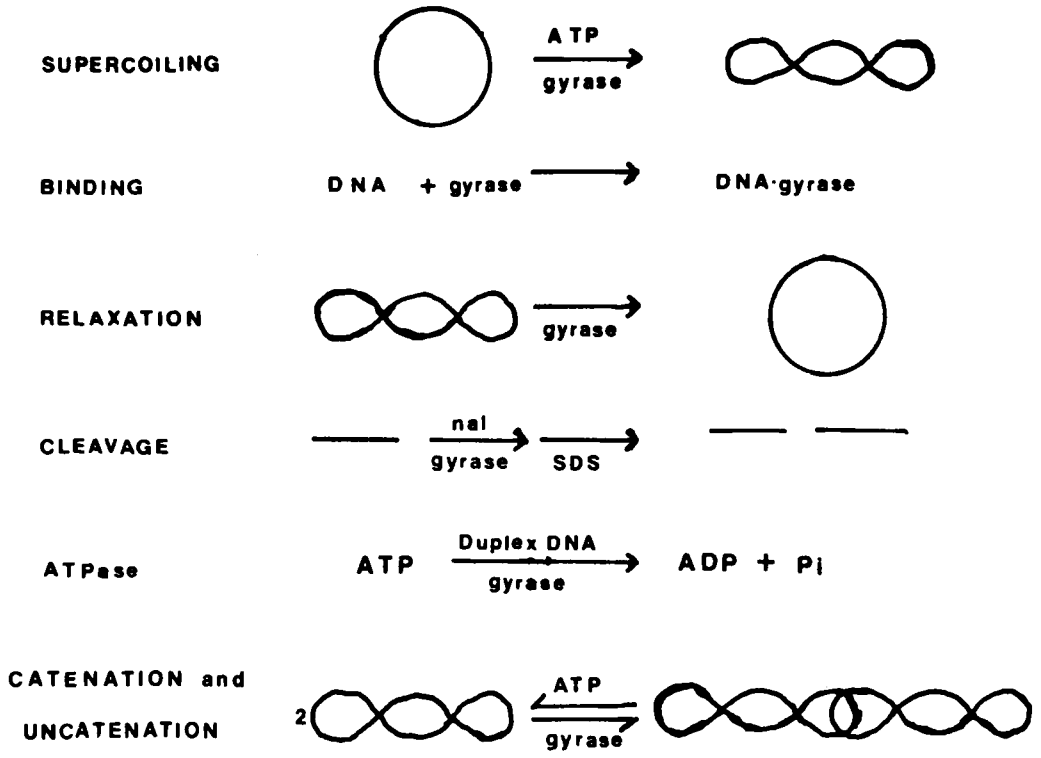

Figure 2.1. The activities of E. coli DNA gyrase.

TABLE 2.1

INHIBITION OF THE ACTIVITIES OF DNA GYRASE BY NOVOBIOCIN AND NALIDIXIC ACID

Activity	Inhibited by Novobiocin	Inhibited by Nalidixic Acid
1. Supercoiling	yes	yes
2. Binding	no	no
3. Relaxation	no	yes
4. Cleavage	no	no
5. ATPase	yes	no
6. Catenation and uncatenation	yes	yes

and introducing a counterposing negative supercoil; (ii) gyrase introduces a double-stranded break in one segment of the DNA, then passes the other segment through the break thus inverting the sign; (iii) the break is then resealed. Figure 2.2 demonstrates the sign inversion process.

E. TOPOISOMERASE ACTIVITIES OF GYRASE

Equimolar amounts of subunit A and B are needed to reconstitute both supercoiling and relaxing activities of DNA gyrase, however highly purified supercoiling activity has reduced relaxing activity. This was explained by the identification of topoisomerase II' (3). It is constructed from subunit A and a 50,000 dalton protein called ν. There is evidence that ν exist normally in the cell and is very similar to subunit B. Among E. coli topoisomerases, topoisomerase II' alone relaxes positive supercoils as efficiently as it relaxes negative supercoils. Topoisomerase II' has no negative supercoiling activity and is not affected by either ATP or novobiocin, however it closely resembles DNA gyrase in its other properties, including sensitivity to oxolinic acid, stabilization of positive supercoiling, double-stranded cleavage at the same DNA sites, and catenation and uncatenation of DNA rings (7).

DNA synthesis directed by almost all bacteriophage is strikingly reduced by inhibition of topoisomerase II and topoisomerase II'. However, the reductions of T4-infected

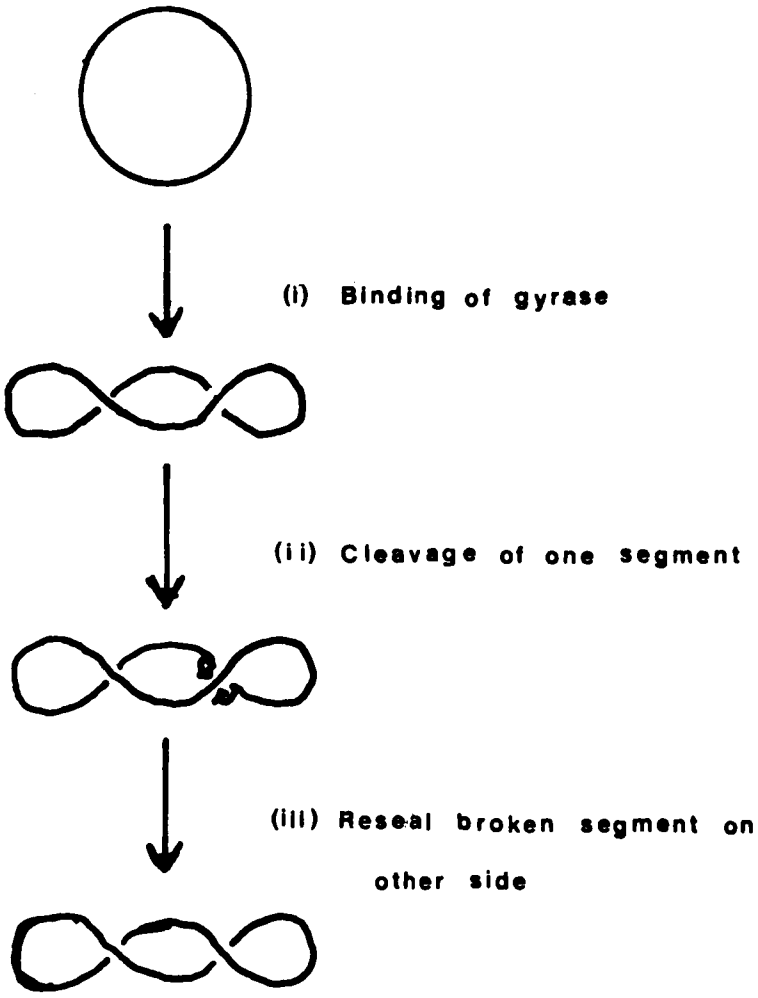

Figure 2.2. The sign inversion model for enzymatic supercoiling of DNA.

cells is only about five-fold (22). Mutants in T4 genes 39, 52, or 60 are totally dependent on the host topoisomerase (27). The products of these three genes are protomers of a T4 topoisomerase described by Stetler et al. (40). T4 topoisomerase requires ATP to relax positive or negative supercoils and has no supercoiling activity. High levels of T4 topoisomerase catenate supercoiled DNA intramolecularly in an ATP-independent reaction, while in the presence of ATP the same enzyme can unknot topologically knotted DNA (7). Gyrase and T4 topoisomerase probably can perform the same function in T4 metabolism since each enzyme spares the requirement for the other (7).

F. PHYSIOLOGICAL ROLE OF GYRASE

Specific drugs, structural gene mutations, and *in vitro* DNA replication and recombination systems have been used to study the functions of DNA gyrase. The results of these investigations have indicated a widespread importance of DNA gyrase in DNA metabolism (7).

There is now good evidence that gyrase is responsible for introducing at least most of the negative supertwist *in vivo* (7). Gyrase inhibitors block supercoiling of infecting phage λ DNA (11,13,16) and drastically reduce the superhelicity of the folded *E. coli* chromosome (9,25,48).

The most striking physiological effect of inactivation of gyrase is the complete inhibition of DNA replication

This inhibition occurs after treatment with nalidixic acid or coumermycin A_1 (6) and temperature shift upwards of a gyrA temperature-sensitive mutant (22,23). The rapidity of the inhibition implies a role in the elongation phase of replication (22).

Supercoiling of the DNA is necessary for the initial nicking by the cisA protein during ϕX174 DNA replication (26). The cisA protein is an endonuclease which is required for ϕX174 DNA replication. Thus gyrase may be involved in the binding of the cisA protein during replication of ϕX174 DNA.

Gyrase is required for other metabolic processes involving DNA as a substrate or template. Inhibitors of gyrase block the transcription of specific operons (10,36, 38,39,49). For example, the expression of early genes of phage N4 require the presence of gyrase (10), while the expression of late genes are insensitive to inhibitors of DNA gyrase. So whether gyrase is involved seems to depend on the promotor involved.

Interest in DNA gyrase comes from its role in λ int-promoted recombination. Supercoiling of λ DNA and int-promoted recombination in vivo and in vitro are effectively blocked by coumermycin A_1 (20,35). The requirement for DNA gyrase in this recombinational process is to provide supertwisted DNA substrate (30). Gyrase may also be involved in other recombinational processes. The

cleavage properties of DNA gyrase has led some investigators to suggest a role in the transposition of genetic elements (7).

Hays and Boehmer have obtained evidence that DNA gyrase is involved in recombination and repair (15). They base their study on the restoration of infectivity of ultraviolet-irradiated phage λ DNA. Antagonists of DNA gyrase showed clear inhibition of this process. Therefore, it is likely that certain repair and recombinational pathways are dependent on DNA gyrase (15).

CHAPTER III

MATERIALS AND METHODS

A. DNA SUBSTRATES

ϕX174 (RF1) DNA was purchased from New England Biolabs while the colicin E1 DNA was prepared from E. coli strain JC411 (ColE1) as described by Tanaka and Weisblum (44). Relaxed closed-circular forms of the DNAs were prepared from supercoiled DNAs by using a chicken nicking-closing extract as described by Gellert et al. (12). The chicken nicking-closing extract was prepared from the nuclei of chicken reticulocytes as described by Comerino-Otero and Felsenfeld (5).

B. ASSAY FOR SUPERCOILING ACTIVITY

The assay for DNA gyrase employed in this study 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 $MgCl_2$, 1.5 mM dithiothreitol, 1.5 mM spermidine-HCl, 1.5 mM ATP, 5 µg bovine serum albumin (BSA), 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 colicin E1 DNA or relaxed closed-circular ϕX174 DNA. The reaction

mixture was incubated at 37°C for 60 min. The reaction was stopped by addition of 5 µl of 0.25 M ethylenediaminetetraacetate (EDTA), and the solution was extracted with an equal volume of chloroform-isoamyl alcohol (24:1 vol/vol). 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 (17). The gels were then stained with a solution of 0.5 µg ethidium bromide per ml of H_2O for 30 min, destained for 30 min in H_2O, and photographed with a Polaroid MP-4 camera using Polaroid type 57 film and a UV-filter (17).

C. PREPARATION OF GYRASE SAMPLES

P. aeruginosa strain RM40 (pur-600, trp-6, met-28, lys-56, str-901) was grown, harvested, and lysed as described in Part I Materials and Methods. All subsequent operations in the purification of the enzyme were carried out at 4°C. Protein determinations were as described in Part I Materials and Methods. Cell debris and DNA were removed by centrifugation for 30 min at 35,000xg. The supernatant fluid (fraction I, 29 ml, 284 mg protein) was treated with a 5% (wt/vol) solution of streptomycin sulfate (Sigma Chemical Co.) prepared in water. The streptomycin solution was added in a proportion of 1 ml/425 optical density units at 260 nm. The mixture was stirred for 20 min, and the precipitate which formed was separated by

centrifugation for 20 min at 27,000xg. 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% (wt/vol) glycerol to produce fraction II (29 ml, 174 mg protein).

A DEAE-cellulose (Cellex D from Bio-Rad Laboratories) column (2.5 x 12.5 cm) was equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.1 mM dithiothreitol, 0.1 mM EDTA, and 30% (wt/vol) glycerol (buffer A). Fraction II was made 30% (wt/vol) in glycerol and was layered directly onto this column, followed by a 35 ml wash of buffer A. A 300 ml gradient of 0.05 to 0.3 M NaCl in buffer A followed and the fractions which eluted between 0.1 and 0.2 M NaCl were pooled. Ammonium sulfate was then added to the pooled fractions to a final concentration of 70% (wt/vol). 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% (wt/vol) glycerol (buffer B) to make fraction III (6 ml, 43 mg protein).

Fraction III was layered onto a column of Sephadex G-200 (1.5 x 48 cm; Pharmacia Fine Chemicals) which had been equilibrated in buffer B. The column was eluted with buffer B. Fractions showing gyrase activity were pooled and concentrated with a Millipore Submersible Concentrator to make fraction IV (3 ml, 8 mg protein).

CHAPTER IV

RESULTS AND DISCUSSION

A. PROPERTIES OF P. AERUGINOSA DNA GYRASE

DNA gyrase activity was not detected until fraction IV. This could be due to the presence of an inhibitor or a nuclease which is removed during purification. This made an accurate estimation of the purification impossible. While not homogenous, fraction IV was free of contaminating deoxyribonuclease activity and the protein content had been reduced 36 fold from fraction I. Fraction IV was stored at -20°C in buffer B. The preparation was unstable and activity could only be detected for 3 to 4 weeks under these conditions. This instability may account for the low specific activity observed. All subsequent experiments were done using fraction IV.

Figure 2.3 demonstrates P. aeruginosa DNA gyrase activity from fraction IV on relaxed closed-circular ϕX174 DNA. P. aeruginosa DNA gyrase was found to require ATP and Mg^{++} for supercoiling activity and this activity is inhibited by novobiocin and nalidixic acid. Maximal activity was observed between 0.5 mM and 3 mM ATP. The enzyme is inactivated by heating to 70°C for 5 min. Identical results were obtained when relaxed closed-circular colicin E1 DNA was used as the substrate. These properties

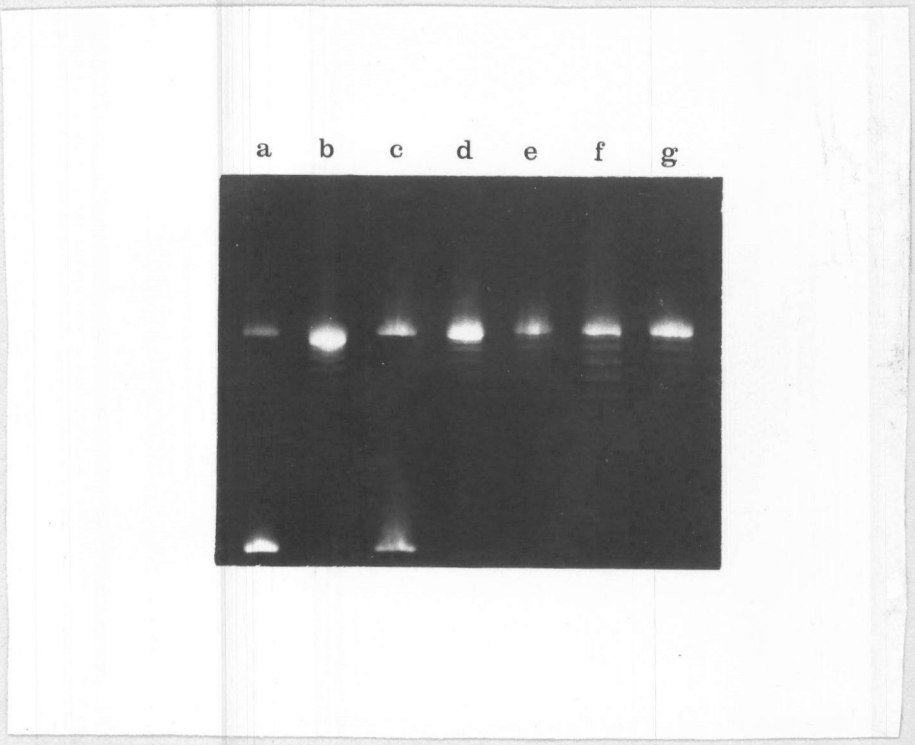

Figure 2.3. Assay of DNA gyrase activity under different conditions. (a) Supercoiled ϕX174 (RF1) DNA (lower band) containing some relaxed circular DNA (upper band). (b) Relaxed closed-circular ϕX174 DNA. (c) Relaxed closed-circular ϕX174 DNA incubated with 30 µl of fraction IV. (d-g) Relaxed closed-circular ϕX174 DNA incubated with 30 µl of fraction IV for 60 min under the following conditions: (d) ATP omitted; (e) Mg^{++} omitted; (f) 750 µg nalidixic acid/ml added; (g) 10 µg novobiocin/ml added. Reactions were carried out as described in the text.

are similar to those found for E. coli DNA gyrase.

While a concentration of 750 µg of nalidixic acid per ml inhibited supercoiling activity of P. aeruginosa DNA gyrase completely, the enzyme retained greater than 50% activity in the presence of 500 µg nalidixic acid per milliliter. This is a much higher concentration of this drug than is required to inhibit the enzyme from B. subtilis (41) and E. coli (43). This finding may explain the fact that P. aeruginosa is resistant to higher concentrations of nalidixic acid (37).

The effects of enzyme sample concentration and time of incubation on the supercoiling of relaxed closed-circular DNA by P. aeruginosa DNA gyrase are shown in Figure 2.4. The number of superhelical twists in the DNA sample increases with enzyme concentration and with incubation time.

The molecular weight of native P. aeruginosa DNA gyrase was estimated by chromatography on Sephadex G-200. Aldolase, catalase, and thyroglobulin (purchased from Sigma Chemical Co.) were used as molecular weight standards. P. aeruginosa DNA gyrase elutes at a position corresponding to an approximate molecular weight of 360,000 shown in Figure 2.5. This is similar to the molecular weight of 350,000 reported for the native form of E. coli DNA gyrase (31).

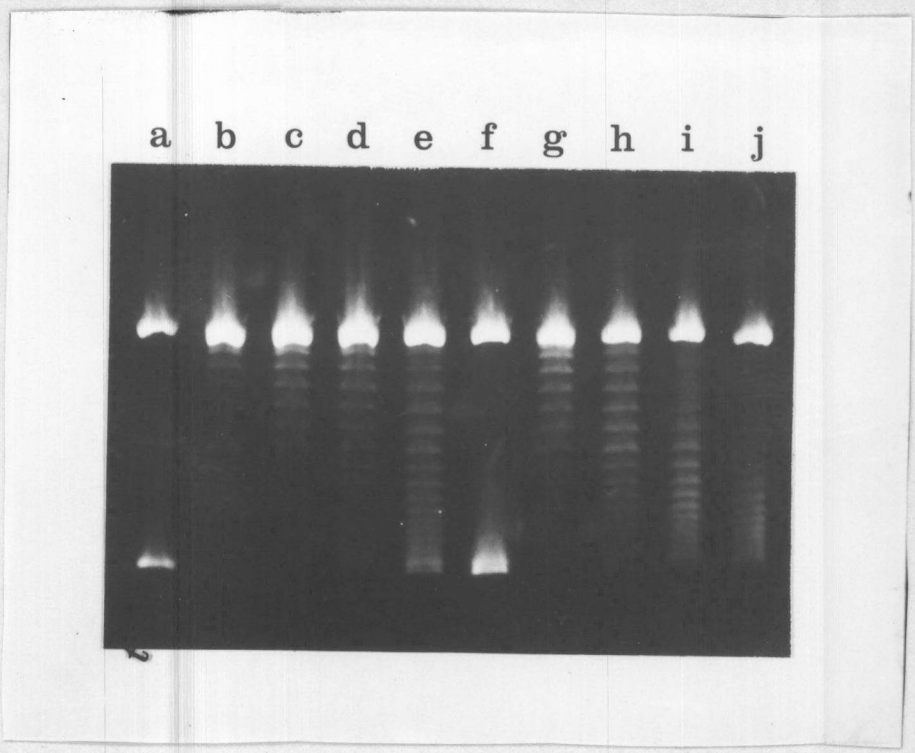

Figure 2.4. Dependence of DNA gyrase supercoiling activity on enzyme concentration and time of incubation. (a) Supercoiled ϕX174 (RF1) DNA (lower band) containing relaxed circular DNA (upper band). (b) Relaxed closed-circular ϕX174 DNA. (c-f) Relaxed closed-circular ϕX174 DNA incubated for 60 min with various amounts of fraction IV: (c) 1 µl; (d) 2 µl; (e) 10 µl; (f) 20 µl. (g-j) Relaxed closed-circular ϕX174 DNA incubated with 10 µl of fraction IV for various times: (g) 5 min; (h) 15 min; (i) 30 min; (j) 60 min. Reactions were carried out as described in the text.

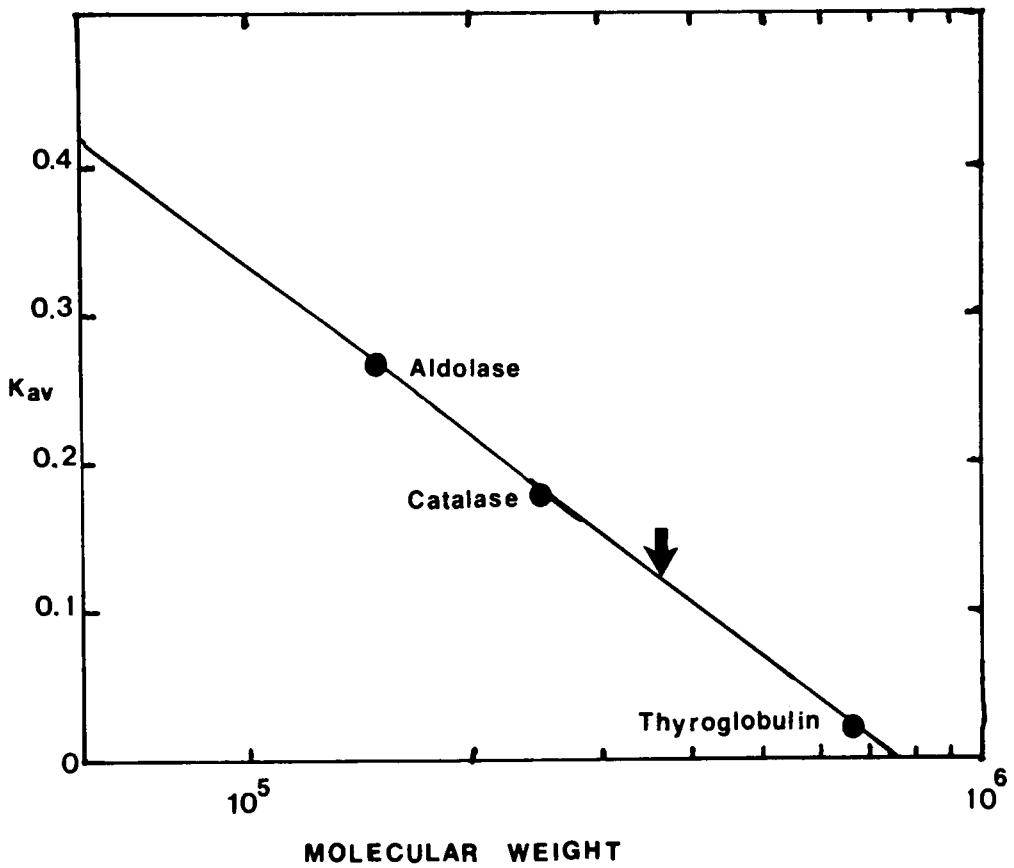

Figure 2.5. Molecular weight determination of P. aeruginosa DNA gyrase by Sephadex G-200 chromatography. The Sephadex G-200 column was as described in the text for fraction IV. Each fraction was assayed from the column as described in the text. Aldolase (MW: 149,000), catalase (MW: 247,000), and thyroglobulin (MW: 669,000) were eluted through the column as molecular weight markers.

B. IDENTIFICATION OF A RELAXING ACTIVITY

E. coli DNA gyrase can relax supercoiled DNA by a nicking-closing activity when ATP is omitted from the reaction mixture (34). In earlier investigation, it was thought that P. aeruginosa DNA gyrase also had a nicking-closing activity, but upon close examination the supercoiling activity could be separated from the relaxing activity during Sephadex G-200 chromatography (Figure 2.6). This relaxing activity has not been described in P. aeruginosa. There does not appear to be any association between DNA gyrase and the relaxing activity which appears in Figure 2.6.

C. ACTIVITIES NOT FOUND IN P. AERUGINOSA GYRASE

E. coli DNA gyrase can introduce double-stranded breaks into DNA in the presence of nalidixic acid or oxolinic acid when incubation is followed by the addition of SDS to the reaction mixture (14,43). It has not been possible to demonstrate either this activity or the relaxing activity described above to be associated with P. aeruginosa DNA gyrase using either the reaction mixture described here or the reaction mixture for the E. coli enzyme (14). It is possible that the reaction conditions employed did not permit the detection of the relaxation or the double-strand cleavage of supercoiled DNA. Recently Kikuchi and Nash (21) have shown that the product of the

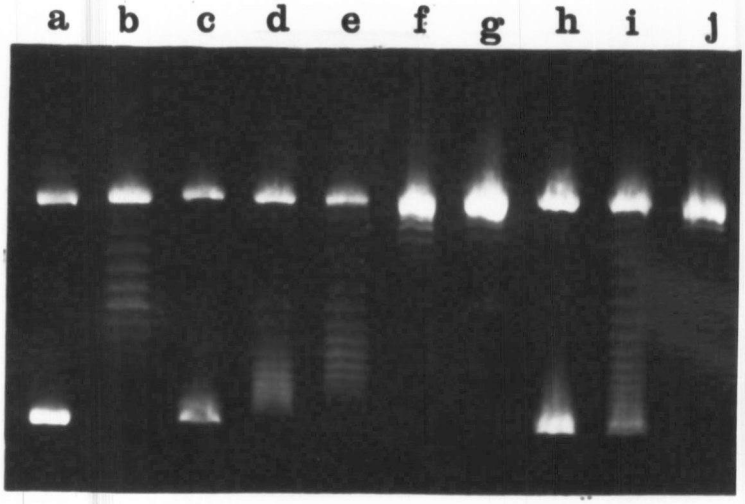

Figure 2.6. Relaxing and supercoiling activity from fraction IV Sephadex G-200 column. (a-e) Supercoiled ϕX174 (RF1) DNA (lower band) containing some open-circular DNA (upper band) incubated with 50 µl of various pooled samples from the Sephadex G-200 column in the absence of ATP: (a) no sample; (b) samples 17-19; (c) samples 23-24; (d) samples 25-26; (e) samples 32-36. (f-j) Relaxed closed-circular ϕX174 DNA incubated with 50 µl of various pooled samples from the Sephadex G-200 column in the presence of ATP: (f) no sample; (g) samples 17-19; (h) samples 23-25; (i) samples 25-26; (j) samples 32-36. Reactions were carried out as described in the text.

lambda *int* gene has a nicking-closing activity which is inhibited by spermidine and Mg^{++}. The removal of the spermidine and Mg^{++} from my reaction mixture did not produce a nicking-closing activity. The rate of relaxation of supercoiled DNA by DNA gyrase from *E. coli* (34) and *B. subtilis* (41) is much lower than the rate of supertwisting. Several explanations are possible. Peebles et al. (34) suggest that the nicking-closing activity of DNA gyrase may be stimulated by ATP and therefore coupled to supercoiling of the DNA substrate. In addition, Stetler et al. (40) have demonstrated that a specific T4-induced protein complex has a nicking-closing activity which is ATP-dependent. It is possible that the nicking-closing activity of *P. aeruginosa* DNA gyrase may also require or be stimulated by ATP.

D. PROSPECTIVES

If *P. aeruginosa* DNA gyrase has a relaxing activity and a double-stranded cleavage activity, it was not detected under the conditions used here. However, the relaxing activity separated from DNA gyrase on the Sephadex G-200 column may have some relationship to DNA gyrase, since preliminary evidence shows that this activity is inhibited by novobiocin. Further purification and comparing isolated subunits to determine if they share common subunits may indicate if there is any relationship between these two activities.

Whether DNA gyrase is involved in the establishment of lysogeny and recombination in P. aeruginosa is not known. Mutant strains deficient in recombination and/or the establishment of lysogeny should be examined for the levels of inhibition by nalidixic acid and novobiocin. A difference in these inhibition levels to those found in the parent strains may indicate an altered gyrase.

One mutant strain of P. aeruginosa, isolated by Miller and Ku (29), which is deficient in the processes of recombination and the establishment of lysogeny appears upon preliminary examination to have a greater resistance to novobiocin than its parent (unpublished observation). Further examination of DNA gyrase from this strain should be undertaken to determine if this is due to an altered gyrase.

Examination of gyrase in other mutant strains deficient in recombination and the establishment of lysogeny may show differences between gyrase from the mutant strain and the wild type. If differences are found, it would indicate that gyrase is involved in the establishment of lysogeny or in genetic recombination.

CHAPTER V

CONCLUSIONS

Part I of this study described the nucleases from P. aeruginosa. Some nucleases have been shown to be involved in the processes of genetic recombination in some bacteria and are probably also involved in genetic recombination in P. aeruginosa. They could play a role in genetic recombination in the preparation of DNA substrates by partially degrading DNA. The partially degraded DNAs could be used as substrates for genetic recombination. Nucleases could also play a role in genetic recombination by degrading excess DNA during the recombinational process. PaeExoIX may be the nuclease which carries out some of these functions.

Unlike nucleases which seem to have limited roles in DNA metabolism, DNA gyrase seems to be involved in most all processes involving DNA metabolism. Therefore, it would not be surprising to find that DNA gyrase is also involved in genetic recombination. DNA gyrase would probably be involved in forming the correct DNA structure for the recombinational process to be carried out. The superhelical structure of DNA may be important in order for other enzymes involved in recombination to carry out their functions. Therefore, P. aeruginosa DNA gyrase could form

a supercoiled DNA structure which may be necessary for genetic recombination to proceed.

LIST OF REFERENCES

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1. Bauer, W. R. 1978. Structure and reactions of closed duplex DNA. Annu. Rev. Biophys. Bioeng. 7:287-313.

2. Brown, P. O. and N. R. Cozzarelli. 1979. A sign inversion mechanism for enzymatic supercoiling of DNA. Science 206:1081-1083.

3. Brown, P. O., C. L. Pebles, and N. R. Cozzarelli. 1979. Topoisomerase from E. coli related to DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. 76:6110-6114.

4. Champoux, J. J. 1978. Proteins that affect DNA conformation. Annu. Rev. Biochem. 47:449-479.

5. Comerini-Otero, R. D. and G. Felsenfeld. 1977. Supercoiling energy and nucleosome formation: the role of the arginine-rich histone kernel. Nucl. Acid Res. 4:1157-1181.

6. Cozzarelli, N. R. 1977. The mechanism of action of inhibitors of DNA synthesis. Annu. Rev. Biochem. 46:641-668.

7. Cozzarelli, N. R. 1980. DNA gyrase and the supercoiling of DNA. Science 207:953-960.

8. DeWyngaert, M. A. and D. C. Hinkle. 1979. Involvement of DNA gyrase in replication and transcription of bacteriophage T7 DNA. J. Virol. 29:529-535.

9. Drlica, K. and M. Snyder. 1978. Superhelical Escherichia coli DNA: relaxation by coumermycin. J. Mol. Biol. 120:145-154.

10. Falco, S. C., R. Zivin, and L. B. Rothman-Denes. 1978. Novel template requirements of N4 virion RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 75:3220-3223.

11. Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. Tomizana. 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. U.S.A. 74:4772-4776.

12. Gellert, M., K. Mizuuchi, M. H. O'Dea, and H. A. Nash. 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. U.S.A. 73:3872-3876.

13. Gellert, M., K. Mizuuchi, M. H. O'Dea, H. Ohmori, and J. Tomizawa. 1979. DNA gyrase and DNA supercoiling. Cold Spring Harbor Symp. Quant. Biol. 43:35-40.

14. 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. U.S.A. 73:4474-4478.

15. Hays, J. B. and S. Boehmer. 1978. Antagonists of DNA gyrase inhibit repair and recombination of UV-irradiated phage λ. Proc. Natl. Acad. Sci. U.S.A. 75:4125-4129.

16. 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. U.S.A. 75:1773-1777.

17. Hinkle, N. F. and R. V. Miller. 1979. pMG7-mediated restriction of Pseudomonas aeruginosa phage DNAs is determined by a class II restriction endonuclease. Plasmid 2:387-393.

18. Holloway, B. W., V. Krishnapillai, and A. F. Morgan. 1979. Chromosomal genetics of Pseudomonas. Microbiol. Rev. 43:73-102.

19. Itoh, T. and J. Tomizawa. 1977. Involvement of DNA gyrase in bacteriophage T7 DNA replication. Nature 270:78-80.

20. Kikuchi, Y. and H. Nash. 1978. Integrative recombination of bacteriophage λ: requirement for supertwisted DNA in vivo and characterization of Int. Cold Spring Harbor Symp. Quant. Biol. 43:1099-1109.

21. Kikuchi, Y. and H. Nash. 1979. Nicking-closing activity associated with bacteriophage λ int gene product. Proc. Natl. Acad. Sci. U.S.A. 76:3760-3764.

22. Kreuzer, K. N. and N. R. Cozzarelli. 1979. Escherichia coli mutants thermosensitive for deoxyribonucleic acid replication, transcription, and bacteriophage growth. J. Bacteriol. 140: 424-435.

23. Kreuzer, K. N., K. McEntee, A. P. Geballe, and N. R. Cozzarelli. 1978. Lambda transducing phages for the nalA gene of Escherichia coli and conditional lethal nalA mutations. Mol. Gen. Genet. 167:129-137.

24. Liu, L. F., C-C. Liu, and B. M. Alberts. 1980. Type II DNA topoisomerases: enzymes that can unknot a topologically knotted DNA molecule via a reversible double-strand break. Cell 19:697-707.

25. Liu, L. F. and J. C. Wang. 1978. Micrococcus luteus DNA gyrase: active components and a model for its supercoiling of DNA. Proc. Natl. Acad. Sci. U.S.A. 75:2098-2102.

26. Marian, K. J., J. Ikeda, S. Schlagman, and J. Hurwitz. 1977. Role of DNA gyrase in ϕX replicative-form replication in vitro. Proc. Natl. Acad. Sci. U.S.A. 74:1965-1968.

27. McCarthy, D. 1979. Gyrase-dependent initiation of bacteriophage T4 DNA replication: interactions of Escherichia coli gyrase with novobiocin, coumermycin and phage DNA-delay gene products. J. Mol. Biol. 127:265-283.

28. Miller, R. V. and A. J. Clark. 1976. Purification and properties of two deoxyribonucleases of Pseudomonas aeruginosa. J. Bacteriol. 127:794-802.

29. Miller, R. V. and C.-M. C. Ku. 1978. Characterization of Pseudomonas aeruginosa mutants deficient in the establishment of lysogeny. J. Bacteriol. 134: 375-383.

30. Mizuuchi, K., M. Gellert, and H. A. Nash. 1978. Involvement of supertwisted DNA in integrative recombination of bacteriophage lambda. J. Mol. Biol. 121:375-392.

31. 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. U.S.A. 75:5960-5963.

32. Morrison, A. and N. R. Cozzarelli. 1979. Site-specific cleavage of DNA by E. coli DNA gyrase. Cell 17:175-184.

33. Nash, H. A., K. Mizuuchi, R. A. Weisberg, Y. Kikuchi, and M. Gellert. 1977. Integrative recombination of bacteriophage λ--the biochemical approach to DNA insertions, pp. 363-373. In A. I. Bukhari et al. (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, New York.

34. Peebles, C. L., N. P. Higgins, K. N. Kreuzer, A. Morrison, P. O. Brown, A. Sugino, and N. R. Cozzarelli. 1978. Activities of Escherichia coli DNA gyrase. Cold Spring Harbor Symp. Quant. Biol. 43:41-52.

35. Pollock, T. J. and K. Abremski. 1979. DNA without supertwist can be an in vitro substrate for site-specific recombination of bacteriophage λ. J. Mol. Biol. 131:651-654.

36. Sanzey, B. 1979. Modulation of gene expression by drugs affecting deoxyribonucleic acid gyrase. J. Bacteriol. 138:40-47.

37. Sherris, J. C. 1974. Future needs, pp. 439-442. In E. H. Lennette, H. Spaulding, and J. P. Truand (ed.), Manual of clinical microbiology, 2nd ed. American Society of Microbiology, Washington, D. C.

38. Shuman, H. and M. Schwartz. 1975. The effect of nalidixic acid on the expression of some genes in Escherichia coli K-12. Biochem. Biophys. Res. Commun. 64:204-209.

39. Smith, C. L., M. Kubo, and F. Imamoto. 1978. Pre-motor-specific inhibition of transcription by antibiotics which act on DNA gyrase. Nature 175:420-423.

40. Stetler, G. L., G. J. King, and W. M. Huang. 1979. T4 DNA delay, proteins required for specific DNA replication, form a complex that has ATP-dependent DNA topoisomerase activity. Proc. Natl. Acad. Sci. U.S.A. 76:3737-3741.

41. Sugino, A. and K. F. Bott. 1980. Bacillus subtilis deoxyribonucleic acid gyrase. J. Bacteriol. 141: 1331-1339.

42. Sugino, A., N. P. Higgins, P. O. Brown, C. L. Peebles and N. R. Cozzarelli. 1978. Energy coupling in DNA gyrase and the mechanism of action of novobiocin. Proc. Natl. Acad. Sci. U.S.A. 75:4838-4842.

43. 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. U.S.A. 74:4767-4771.

44. Tanaka, T. and B. Weisblum. 1975. Construction of a colicin E1-R factor composite plasmid *in vitro*: means for amplification of deoxyribonucleic acid. J. Bacteriol. 121:354-362.

45. Taylor, D. E. and J. G. Levine. 1979. Characterization of a plasmid mutation affecting maintenance, transfer and elimination by novobiocin. Molec. Gen. Genet. 174:127-133.

46. Wang, J. C. 1971. Interaction between DNA and an *Escherichia coli* protein ω. J. Mol. Biol. 55: 523-533.

47. Wang, J. C. 1979. DNA: bihelical structure, supercoiling and relaxation. Cold Spring Harbor Symp. Quant. Biol. 43:29-33.

48. Worcel, A. and E. Burgi. 1972. On the structure of the folded chromosome of *Escherichia coli*. J. Mol. Biol. 71:127-147.

49. Yang, H-L., K. Heller, M. Gellert, and G. Zubay. 1979. Differential sensitivity of gene expression *in vitro* to inhibitors of DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. 76:3304-3308.

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