Purification and Properties of Two Deoxyribonucleases of *Pseudomonas aeruginosa*

ROBERT V. MILLER[1] AND ALVIN J. CLARK*

Department of Molecular Biology, University of California, Berkeley, California 94720

A survey of the major deoxyribonucleases in *Pseudomonas aeruginosa* strain PAO was undertaken. Two activities predominated in Brij-58 lysates of this organism. These have been purified from contaminating nuclease activities, and some of their properties have been elucidated. The first was a nuclease that degraded heat-denatured deoxyribonucleic acid (DNA) to mono- and dinucleotides. The activity of this enzyme was confined to single-stranded DNA, and 100% of the substrate was hydrolyzed to acid-soluble material. The Mg^{2+} optimum is low (1 to 3 mM), and the molecular weight is 6×10^4. The second predominant activity was an adenosine 5'-triphosphate (ATP)-dependent deoxyribonuclease. This enzyme had an absolute dependence on the presence of ATP and double-stranded DNA for activity. The activity was greatly enhanced at Mg^{2+} concentrations of approximately 10 mM. Five moles of ATP was consumed for each mole of phosphodiester bonds cleaved. The acid-soluble products of the reaction consisted of short oligonucleotides from one to six bases in length. Only 50% of the double-stranded DNA was rendered acid soluble in a limit digest. The molecular weight of this enzyme is 3×10^5. The observation of these enzymes in *P. aeruginosa* is consistent with the possibility that recombinational pathways similar to those of *Escherichia coli* are operating in this organism.

Pseudomonas aeruginosa engages in conjugational and transductional genetic recombination as does *Escherichia coli* (13). One approach to determine whether or not the pathways of recombination in these two species are similar is that of comparative enzymology. According to this approach, enzymes with similar substrate specificities and modes of action are expected to participate in similar metabolic roles. For genetic recombination, the pathways of intermediates and the characteristics of the enzymes are not as completely worked out as they are for amino acid biosynthesis, for example. Nonetheless, in the long run, the comparative approach should be as fruitful in the area of genetic recombination as it is in more traditional areas of intermediary metabolism.

In *E. coli*, at present, three deoxyribonucleic acid (DNA) exonucleases are associated with genetic recombination: exonuclease I (ExoI) (17), exonuclease V (ExoV) (2, 5, 24), and exonuclease VIII (ExoVIII) (3, 16). A hypothesis involving three pathways of genetic recombination has been proposed to summarize the phenotypes of mutants in which these enzymes are affected (8, 9). With this in mind, we began a search for analogues of these three *E. coli* en-

[1] Present address: Department of Microbiology, University of Tennessee, Knoxville, Tenn. 37916.

zymes in extracts of *P. aeruginosa*. Salient characteristics were used in the original screening. ExoV is active on linear native DNA only when adenosine 5'-triphosphate (ATP) is added. ExoVIII is active on linear native DNA in the absence of ATP. ExoI is active on linear denatured DNA in the absence of ATP. Hence, nuclease activity in crude extracts under these three conditions was determined. At present, there are no other ATP-dependent activities in *E. coli* that can be mistaken for ExoV. There are, however, other enzymes in crude extracts of *E. coli* that mimic the activities of ExoVIII and ExoI, which we planned to assay. Exonuclease III (ExoIII) also acts on linear native DNA in the absence of ATP (28), as does the exonuclease VI (5'-3' exonuclease) activity of DNA polymerase I (27). Exonuclease VII (ExoVII) acts on linear denatured DNA in the absence of ATP (7), as does the exonuclease II (3'-5' exonuclease) activity of DNA polymerase I (27). To characterize any detected ATP-independent activity and determine which of the enzymes of *E. coli* it most closely resembles, we planned to resort to fractionation procedures and several diagnostic tests on purified material. In the case of the ATP-dependent activity, purification was necessary to determine whether the *P. aeruginosa* counterpart had the substrate versatility of ExoV.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strain JC9006 was used throughout. This is an adenine-requiring (i.e., Ade⁻) derivative of strain PAO and is otherwise wild type.

DNA. Labeled and unlabeled *P. aeruginosa* and *E. coli* DNAs were prepared by the method of Lehman (19), except that labeling the DNA in *P. aeruginosa* was performed as described by Pemberton and Clark (25). Cross-linked DNA was prepared by the method of Cole and Zusman (10) as modified by Karu and Linn (14), using 4,5',8-trimethylpsoralen. fd phage DNA was the kind gift of Stuart Linn. Single-stranded bacterial DNA was prepared by heating native DNA in a boiling-water bath for 10 min in 0.14 M NaCl-0.01 M sodium citrate at a concentration of 150 nmol/ml and then cooling the mixture in an ice water-sodium chloride bath until the temperature had fallen below 10°C.

Enzymes. Alkaline phosphatase and lysozyme were obtained from Worthington Biochemicals Corp. Catalase was obtained from Boehringer-Mannheim Biochemical. Partially purified ExoI was the kind gift of Stuart Linn.

Other materials. [³H]adenine and [³H]thymidine were from Schwarz/Mann Co. [³²P]PO₄ and γ-[³²P]ATP were purchased from ICN Pharmaceuticals Corp. Diethylaminoethyl-cellulose was Cellex-D from Bio-Rad Laboratories. Sephadex G200 was from Pharmacia Fine Chemicals, Inc., and DNA cellulose was prepared by the method of Goldmark and Linn (12), using Bio-Rad Munktell 410 cellulose. Bovine serum albumin (BSA) and yeast nucleic acid were from Calbiochem, and thyroglobulin was obtained from Sigma Chemical Co. Streptomycin sulfate was from Sigma Chemical Co. 4,5',8-Trimethylpsoralen was the kind gift of A. Karu.

Nuclease assay with duplex DNA. Nuclease activity on duplex DNA was measured by the release of acid-soluble fragments from native DNA. The standard reaction mix (0.5 ml) included tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride (10 mM, pH 8.0), β-mercaptoethanol (10 mM), MgSO₄ (10 mM), ATP (40 μM), and 6.6 nmol of [³H]DNA (ca. 10⁴ cpm/nmol from either *P. aeruginosa* or *E. coli*). Usually, reactions were carried out for 20 min at 37°C. After that time they were placed on ice and 0.2 ml of a yeast nucleic acid solution (2 mg/ml dissolved in 50 mM Tris-hydrochloride at pH 8.0) was added, followed by 0.7 ml of 10% trichloroacetic acid. The acid-precipitable material was sedimented by centrifugation for 10 min at 12,000 × *g*. A sample (0.2 ml) of the supernatant fluid was counted in a Packard scintillation counter as described below. One unit of enzyme converts 1 nmol of nucleotides to acid-soluble products in 20 min at 37°C. The assay is linear up to a level of 0.8 unit of enzyme for 30 min. The enzyme is equally active on *E. coli* DNA and on *P. aeruginosa* DNA prepared from strain JC9006. Assays for nuclease activity on double-stranded DNA in the absence of ATP were carried out under exactly the same conditions, except no ATP was added to the reaction mix.

Nuclease assay with denatured DNA. Nuclease activity on single-stranded DNA was measured by the release of acid-soluble fragments from heat-denatured and quick-cooled DNA. The standard assay mix (0.3 ml) contained Tris-hydrochloride (50 mM, pH 8.0), β-mercaptoethanol (1.7 mM), MgSO₄ (1.7 mM), and ³H-labeled, heat-denatured *E. coli* DNA (30 nmol). After 20 min at 37°C, the reaction was stopped and acid-solubilized nucleotides were assayed in the same manner as in the nuclease assay for double-stranded DNA. The assay was linear from 0.1 to 0.8 unit. One unit of enzyme hydrolyzed 1 nmol of nucleotide to acid-soluble products in 20 min at 37°C.

Endonuclease assay. Endonuclease activity was measured in 0.15 ml of a solution containing 5 μmol of β-mercaptoethanol, 4 μmol of dithiothreitol, 60 μmol of MgCl₂, 5 units of ExoI, and 4 nmol of fd phage DNA (³H-labeled), a closed circular, single-stranded DNA. The pH of the reaction was adjusted with 50 mM Tris-hydrochloride to pH 7 or to pH 9.5 with 50 mM glycylglycine. The enzyme fraction to be tested was added to start the reaction, which was allowed to proceed at 37°C for 30 min. A 500-μg portion of BSA and 0.25 ml of 7% trichloroacetic acid were added to stop the reaction and precipitate non-digested DNA. Activity was measured as increase in acid-solubilized counts in the reaction mix over the amount released in a control tube containing exonuclease I but no *Pseudomonas* enzyme fraction.

Glycerol gradients. Linear glycerol gradients (20 to 40%) were centrifuged in a Spinco SW50.1 swinging-bucket head at 50,000 rpm (204,000 × *g*, average) for 12 h in an L2-65B ultracentrifuge. Gradients were prepared in 30 mM potassium phosphate buffer (pH 6.8).

Paper chromatography. Samples were spotted on Whatman 3 MM paper and chromatographed by descending elution in 1-propanol–ammonia–water 6:3:1) for 69 h (12). Markers included the four deoxyribonucleoside 5'-monophosphates and (pA)₂, (pA)₃, (pA)₄, (pA)₅, and (pA)₆. After elution, the chromatogram was dried and cut into strips. The strips were cut into 1-cm pieces and counted in the liquid scintillation counter.

Other methods. Liquid scintillation counting was accomplished by addition of 4 ml of scintillation fluid {18.2 g of 2,5-diphenyloxazole, 1.22 g of 1,4-bis-[2-(5-phenyloxazolyl)]benzene, 2,500 ml of Triton X-100, and 4,280 ml of toluene} to 0.2 ml of aqueous-phase sample. Nonaqueous samples were counted in 4 ml of nonaqueous scintillator {12 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis-[2-(5-phenyloxazolyl)]benzene in 3 liters of toluene}. Protein determinations were made by the method of Lowry et al. (21) using BSA as a standard. Concentrations below 0.05 mg/ml were measured by ultraviolet absorbancy at 280 nm. Determination of [³²P]PO₄ released from ATP or DNA was by the Norit adsorption method of Goldmark and Linn (12). In addition to the washes, the Norit was transferred to a planchet so that the percentage of Norit nonadsorbable PO₄ could be determined directly. Samples were counted on a Nuclear-Chicago gas flow counter.

Preparation of cell extracts. (i) Method 1 (survey method). Strains of *P. aeruginosa* PAO were inoculated in 10-ml amounts of M9 medium supplemented with glucose and Casamino Acids (1) and incubated

with periodic dilution until they were in balanced growth (three generations). Cells were harvested by centrifugation and washed with M9. They were resuspended in 10 ml of 0.85% saline, incubated for 10 min at 37°C, and broken by the Brij-lysozyme method described by Barbour and Clark (2). Cleared lysates were prepared by centrifugation of the broken cell suspension at 12,000 × g for 10 min and decantation of the supernatant fluid.

(ii) **Method 2 (preparative method).** Strain JC9006 was inoculated and grown in 10 liters of Luria complete broth until late log phase (Klett units at 660 nm = 125), and the cells were harvested by centrifugation and resuspended in 200 ml of 0.85% saline. The cells were incubated at 37°C for 10 min and were then reharvested by centrifugation (net weight of cells, 20 to 25 g). Cell lysis was accomplished by modification of a method originally described by Wickner et al. (36). Cells were suspended at a concentration of 0.5 g/ml of 10% sucrose solution prepared in 0.5 M Tris-hydrochloride buffer (pH 7.5). This suspension was then frozen in liquid nitrogen and thawed at room temperature. The volume was measured, and 0.1 ml of lysozyme (2 mg/ml) and 0.1 ml of 1 M NaCl were added for each milliliter of cell suspension. The mixture was allowed to incubate at 0°C on ice for 60 to 75 min. At this point the solution had become quite viscous, due to release of DNA from the lysed cells. All subsequent operations were carried out at 0 to 4°C. Cell debris was removed by centrifugation for 20 min at 35,000 × g. The supernatant fluid was decanted and considered fraction I in the subsequent nuclease fractionation steps.

Fractionation steps used in enzyme purification. (i) **Dihydrostreptomycin precipitation.** Fraction I was treated with 5% (wt/vol) dihydrostreptomycin sulfate solution (freshly prepared). The streptomycin solution was added slowly, with constant stirring, 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 27,000 × g. The precipitate was discarded and the supernatant fluid was considered fraction II.

(ii) **Dialysis.** Fraction II was dialyzed for 4 h against two changes of 50 mM Tris-hydrochloride buffer (pH 8.0) containing 0.1 mM dithiothreitol solution. This treatment usually increased the activity of the ATP-dependent deoxyribonuclease significantly, presumably by the removal or the inactivation of an inhibitor. During dialysis, a precipitate formed that had no detectable nuclease activity. This was removed by centrifugation at 12,000 × g for 10 min. Glycerol was added to the supernatant fluid to a final concentration of 30% to produce fraction III.

(iii) **Diethylaminoethyl-cellulose chromatography.** A diethylaminoethyl-cellulose column (2.5 by 12.5 cm) was equilibrated with 50 mM Tris-hydrochloride (pH 8.0), 0.1 mM dithiothreitol, 30% glycerol (buffer A). Fraction III 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 60 ml/h was maintained. Nuclease activities on both single- and double-

stranded DNA eluted as a single broad peak from this column from 0.10 to 0.17 M NaCl. Fractions containing greater than 33 units of activity per ml on single-stranded DNA were pooled and dialyzed against 0.03 M potassium phosphate buffer (pH 6.8) containing 30% glycerol and 10^{-4} M dithiothreitol (buffer B) for 6 h. The fraction was then concentrated in an Amicon pressure concentrator to 5 ml. (Recovery after concentration was greater than 80%.) The concentrated preparation was designated fraction IV.

(iv) **Sephadex G-200 chromatography.** Fraction IV was layered onto a column of Sephadex G-200, which had been swollen and equilibrated in buffer B. The column dimensions were 2 by 40 cm. The column was then eluted with buffer B. The ATP-dependent activity on double-stranded DNA eluted from this column within the included volume one to two fractions behind blue dextran (fraction Vd). It was virtually free of any activity on single-stranded DNA. The single-stranded activity eluted much later from the column in a molecular weight range between 50,000 and 100,000 (fraction Vs). The two activities were pooled separately.

(v) **DNA-cellulose column chromatography.** Fractions Vd and Vs were subjected separately to DNA-cellulose chromatography. Each fraction was layered on a DNA-cellulose column (0.6 by 5 cm) which had been equilibrated in buffer B. A flow rate of 3 ml/h was maintained. The sample was followed by a 1.5-ml wash of buffer B and a 20-ml gradient of 0.0 to 0.7 M KCl in buffer B. One-milliliter fractions were taken. The nuclease active on single-stranded DNA eluted between 0.16 and 0.21 M KCl (fraction VIs), whereas the ATP-dependent activity on double-stranded DNA eluted from a similar column at 0.28 to 0.31 M KCl (fraction VId). All subsequent studies were done on these fractions unless otherwise stated.

Enzyme abbreviations. DNA exonuclease enzymes have been associated with genetic recombination in *E. coli,* and one can reasonably expect counterparts to occur in other species. When such counterparts are discovered, it will be convenient to have a convention for naming them and abbreviating the names. In the absence of a comprehensive proposal, we consider nuclease enzymes of *E. coli* to be type enzymes and have adopted their names as names of generic enzyme classes. The exonucleases of *E. coli* have been numbered serially with Roman numerals as each has been distinguished from those previously described (20). Thus, for example, we consider there to be a class of enzymes called exonuclease I (ExoI) enzymes and a type enzyme, the ExoI from *E. coli.* To indicate that the ExoI from *E. coli* is a different member of the ExoI class of enzymes from the ExoI that may be found in *P. aeruginosa,* it will be necessary to append to the enzyme class abbreviation an abbreviation of the genus and species of origin. We shall adopt the convention proposed by Smith and Nathans (31), which uses the first letter of the genus name and the first two letters of the species name as an abbreviation. Hence, we shall denote two members of the ExoI class of enzymes as *Eco*ExoI and *Pae*ExoI, with *Eco*ExoI being the type

enzyme. In this abbreviation we have deviated from the convention of Smith and Nathans by prefixing rather than suffixing the genus-species abbreviation. In the case of enzymes determined by phage lambda, it is impossible to follow this convention strictly since phages are not given genus and species names. We therefore shall use the first three letters of the English word for the Greek letter λ, which is the name of the phage. Should a lambda phage gene ever be found to determine an ExoI enzyme, this enzyme would be denoted *Lam*ExoI.

RESULTS

Detection of two nuclease activities and purification of the responsible enzymes. Lysozyme-Brij extracts of four strains of *P. aeruginosa* were surveyed to detect nuclease activity on native and denatured DNA. Very little activity on native DNA was detected (1 to 2 units/mg of protein) unless ATP was added, in which case 10 to 15 times as much activity was observed. On denatured DNA, from 7 to 12 units of activity were observed in the absence of ATP. Essentially the same amount of activity was detected in the presence of ATP.

These findings encouraged us to think that we might be able to purify both an ATP-dependent nuclease active on native DNA and an ATP-independent nuclease active on denatured DNA. To begin the purification, we used a different method of rupturing the cells. Extracts obtained by this freeze-thaw lysis method showed about eight times more ATP-independent activity on native DNA than observed in the extracts produced by the lysozyme-Brij method. Consequently, the stimulation of nuclease digestion was reduced to less than twofold. We reasoned that the difference in the results obtained with the crude extracts might be due to different concentrations of Mg^{2+}, since no Mg^{2+} is added to the medium in which the cells were frozen and thawed, whereas the lyso-

zyme-Brij mixture is made 50 mM in Mg^{2+}. Perhaps 50 mM Mg^{2+} is inhibitory to the ATP-independent activity. In support of this, we found that addition of Mg^{2+} to the extracts produced by the freeze-thaw method reduced the amount of nuclease activity on native DNA detectable in the absence of ATP.

The crude extract produced by the freeze-thaw method (fraction I) was subjected to various fractionation procedures as described above. ATP-independent activity on denatured DNA and ATP-dependent activity on native DNA were followed separately (Table 1). They co-purified until the step involving Sephadex G-200 chromatography. At this point the ATP-dependent activity on native DNA eluted in the fractions immediately behind the blue dextran, whereas the ATP-independent activity on denatured DNA eluted much more slowly. In addition, we found that virtually no activity on denatured DNA was evident either in the presence or the absence of ATP in the fractions eluting early from the Sephadex G-200. Likewise, there was very little ATP-dependent or ATP-independent activity on native DNA, which eluted from the column with the activity on denatured DNA. The two activities were then subjected separately to DNA-cellulose chromatography for the final purification step. The quantitative recoveries of each activity in all the steps are summarized in Table 1.

Properties of the nuclease in fraction VIs. The molecular weight of the nuclease in fraction VIs was estimated to be 6×10^4 by determining its sedimentation constant to be 4.7 in a 20 to 40% (wt/vol) linear glycerol gradient. Thyroglobulin and catalase were included in a parallel gradient as sedimentation velocity standards. The molecular weight was derived using the method of Martin and Ames (22) from the $s_{20,w}$ values so determined.

TABLE 1. *Purification of the nucleases*

Fraction	Vol (ml)	Protein (mg/ml)	Activity on single-stranded DNA	Activity on double-stranded DNA	
				ATP dependent	ATP independent
I. Cleared lysate	57	6.4	68	6	11
II. Streptomycin	59	6.7	73	11	13
III. Dialysate	102	3.2	83	29	12
IV. Diethylaminoethyl concentrate	5	9.4	106	83	64
Vs. Sephadex G-200	5	1.0	880	<1	33
Vd. Sephadex G-200	8	0.5	<1	89	6
VIs. DNA cellulose	3	~0.003	13,200	<1	<1
VId. DNA cellulose	3	0.022	<1	2,010	<1

The enzyme 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 magnesium optimum of the enzyme is 2.5 mM. The enzyme is about 40% active either at 12 mM magnesium or without any added magnesium. It is, however, inhibited by the presence of disodium ethylenediaminetetraacetate.

The nuclease in fraction VIs favors denatured DNA as a substrate (Table 2); no detectable degradation of double-stranded DNA could be detected. The activity is stimulated slightly by the addition of ATP (Table 2).

The products of the nuclease in fraction VIs were determined by obtaining a limit digest of denatured DNA. Five units of activity from fraction VIs were added to a reaction mixture containing 1 nmol of ^{32}P-labeled $E.$ $coli$ DNA that had been heat denatured as described in Materials and Methods. The reaction was allowed to continue for 150 min at 37°C. One additional unit of enzyme was added at 60 and another at 120 min. The reaction mixture was then heated to 90°C for 5 min to stop the reaction and finally cooled on ice. A portion (either 25 or 100 μl) was sampled and trichloroacetic acid precipitated to determine solubilized nucleotides, as described in Materials and Methods. An additional portion of the reaction mixture was treated with 3.7 units of alkaline phosphatase for 30 min at 37°C to remove terminal phosphate. The percentage of ^{32}PO$_4$ releasable by alkaline phosphatase was calculated by determining the Norit nonadsorbable radioactivity, as described in Materials and Methods. The remainder of the preparation was subjected to
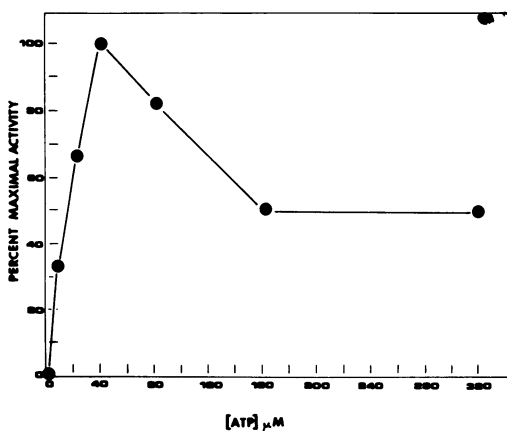
TABLE 2. Properties of nuclease in fraction VIs

Expt no.	DNA substrate	Presence of ATP	Acid solubilized (pmol)	% Activity
1	Denatured	−	380	100
	Native	−	0	<0.03
2	Denatured	−	1,141	100
	Denatured	+	1,294	112

descending paper chromatography, as described in Materials and Methods. One hundred percent of the DNA added to the reaction mixture was acid solubilized during the digestion period. Of the phosphate present, 86% was Norit nonadsorbable, which corresponds to an average chain length of 1.2 nucleotides. Chromatography revealed a slightly longer average chain length of 1.8, with a distribution of oligonucleotides as described in Table 3.

Properties of the nuclease in fraction VId. The molecular weight of the enzyme in fraction VId was estimated to be 3×10^5 from its $s_{20,w}$ of 13.6. The method used was the same as that used to determine the molecular weight of the enzyme in fraction VIs.

The nuclease in fraction VId has a pH optimum between 7.0 and 8.0 and demonstrates greater than 90% activity at the extremes of this range. At pH 6.5, however, the enzyme has only 20% maximal activity, whereas at pH 9.0 it is 75% active. The magnesium optimum is about 10 mM, and 60% maximal activity is shown at 2 and 26 mM magnesium. There is significant activity with no added magnesium. Stimulation of ATP-independent activity in the absence of Mg^{2+} ion observed in crude extracts is not observed with the purified enzyme; the dependence on ATP is absolute at all Mg^{2+} concentrations.

The nuclease activity in fraction VId is observed only in the presence of double-stranded DNA (Table 4). When a sample of fraction VId is incubated with denatured linear DNA or single-stranded circular DNA (fd phage DNA isolated from the virion), no acid solubilization of radioactivity is observed. Endonuclease activity on fd circular single-stranded DNA was tested in the presence and absence of acetylated BSA, because Goldmark and Linn (12) have demonstrated the BSA dependence of the single-stranded DNA endonuclease activity of exonuclease V from $E.$ $coli$ (12). No activity was detectable in the presence of BSA or in its absence. The addition of ATP at a concentration optimal for the activity on double-stranded DNA (see below) did not allow the digestion of closed circular phage DNA nor did the varia-

FIG. 1. Dependence on ATP concentration of the double-stranded DNA nuclease in fraction VId. Reactions were carried out as described in Materials and Methods, except that the concentration of ATP was varied as indicated in the figure.

TABLE 3. *Chain length of digestion products of denatured DNA by the deoxyribonucleases in fractions VIs and VId*

Deoxyribonucleotide spot	% ³²P-labeled nucleotide acid solubilized	
	Fraction VIs	Fraction VId
Mononucleotides	47	5
Dinucleotides	32	36
Trinucleotides	21	26
Tetranucleotides	2	12
Pentanucleotides	0	12
Hexanucleotides	0	9
>Hexanucleotides	0	<1

TABLE 4. *Substrate specificity of the double-stranded DNA nuclease[a]*

DNA substrate	ATP (40 µM)	³H-labeled nucleotide acid solubilized	
		cpm	pmol
Native, linear	+	3,738	349
	−	2	<1
Heat denatured linear	+	0	0
	−	0	0
Closed circular single-stranded (acetylated BSA present)	+	0	0
	−	0	0
Closed circular single-stranded (acetylated BSA absent)	+	0	0
	−	0	0

[a] Assays were as described in Materials and Methods. Background blanks were assayed under each condition and subtracted.

tion of the pH of the reaction mixture from pH 7.0 to 9.0.

To test the ability of the enzyme in fraction VId to attack cross-linked DNA we challenged it with double-stranded DNA, which contained approximately 20 cross-links per nucleotide chain. Karu and Linn (14) have demonstrated the inability of *E. coli* exonuclease V to attack such a substrate. A similar phenomenon has been noted by Pricer and Weissback (26) for λ exonuclease and the exonuclease activity of DNA polymerase I. When challenged, the activity of the *Pseudomonas* deoxyribonuclease was reduced to approximately 6% of the activity exhibited on non-cross-linked native DNA (Table 5). In a molecule that, on the average, has 20 cross-links, one would expect the first cross-link to appear about 5% of the linear length from the end of the molecule. From the activity observed, it would appear that the enzyme is able to bind and degrade the cross-linked DNA until the first cross-link is reached but cannot proceed further along the molecule.

A limit digest of native DNA was carried out to determine the extent of degradation and to estimate the average chain length of the acid-soluble reaction products. One nanomole of ³²P-labeled native DNA was incubated with 5 units of deoxyribonuclease at 37°C for 150 min. Additional units of enzyme were added at 30, 60, and 90 min, and additional ATP (10% of original concentration) was added at 20, 60, 90, and 120 min. At this point kinetic data indicate that the reaction has reached its limit and approximately 56% of the DNA has been solubilized. Portions of the reaction mixture were treated with alkaline phosphatase or chromatographed to determine the chain length of the trichloroacetic acid-solubilized material. Of the total ³²PO₄ present in DNA, 15.5% was susceptible to alkaline phosphatase. Correcting for the fact that only 56% of the reaction was hydrolyzed by the enzyme, this indicated that 28% of the PO₄ groups acid solubilized were 5'-termini and the average chain length of the nucleotide solubilized was about 4. Chromatography of the reaction mix showed a distribution from mono- to hexanucleotides among the acid-solubilized species (Table 3). The average chain length of these oligonucleotides was about 3.

The nuclease in fraction VId is inhibited by concentrations of ATP higher than 40 µM (Fig. 1); consequently, it shows maximum activity at 40 µM ATP. The degradation of ATP is in most cases dependent upon the presence of a degradable DNA species (Table 6). The one exception is in the presence of cross-linked DNA. In this case, whereas the DNA is attacked to a much reduced extent, ATP is hydrolyzed at approximately the same rate as in the presence of native DNA.

To determine the number of ATP molecules hydrolyzed per phosphodiester bond cleaved, γ-³²P-labeled ATP was included in a digestion of native ³H-labeled DNA. Increase in Norit nonadsorbable phosphate was taken as a measure of the amount of ATP hydrolyzed. DNA hydro-

TABLE 5. *Reactivity of ATP-dependent, double-strand-specific deoxyribonuclease on cross-linked DNA[a]*

DNA substrate	³H-labeled nucleotide acid solubilized		% Activity
	cpm	pmol	
Native	19,180	1.80	100
Cross-linked	532	0.11	6

[a] Reactions were carried out as described in Materials and Methods using native or cross-linked DNA. Background counts per minute have been subtracted.

TABLE 6. *Activation of adenosine triphosphatase by various DNAs[a]*

DNA present	PO₄ release (nmol)
None	0.04
Heat denatured linear	0.04
Native	1.46
Cross-linked native	1.88

[a] Assays were carried out in the presence of γ-³²PO₄-labeled ATP as described in Materials and Methods. PO₄ released was determined by the Norit adsorption method.

lyzed was measured by determining acid-solubilized ³H-labeled nucleotide. On the average, 1.43 mol of ATP was hydrolyzed for each mole of nucleotide trichloroacetic acid solubilized. Using 3.5 as the average chain length of the acid-soluble products of the reaction, we calculate that approximately 5 mol of ATP is used for each mole of phosphodiester bonds broken in the degradation of DNA.

Changes in ATP-dependent nuclease activity upon phage infection. Exonuclease V in *E. coli* is inactivated by the gene product of the gamma gene of lambda phage (32). Recently, a similar inactivation has been shown after infection of this bacterium by other coliphage (29). In a first attempt to identify the physiological similarities between the ATP-dependent double-stranded DNA nuclease activity of *P. aeruginosa* and exonuclease V of *E. coli,* the effects of phage infection and lysogenization on the *Pseudomonas* deoxyribonuclease were investigated. Strains were grown to a concentration of approximately 10⁹ cells/ml in Luria complete medium and infected either with phage at a multiplicity of 5 plaque-forming units/cell or diluted with an equal volume of medium not containing phage. Adsorption was allowed for 20 min at room temperature, and the cultures were diluted 1 to 10 and incubated at 37°C for 20 min (approximately 1/3 of the eclipse period for these phages). The cultures were lysed by method I (see Materials and Methods), and cleared lysates were prepared as described above. The ATP-dependent double-stranded DNA nuclease activity was tested in two strains, each infected separately with three different temperate phages (D3, G101, and F116). Activity levels greater than or equal to those in uninfected cells were measured. Either the phages do not irreversibly inhibit the host enzyme or they do and determine a compensating phage enzyme. Alternatively, there may be reversible in vivo inhibition of the enzyme.

DISCUSSION

In a preliminary test of the DNA nuclease activities of crude extracts of strains of *P. aeruginosa* isolate PAO, we detected three different activities: (i) ATP-independent activity on linear native DNA; (ii) ATP-dependent activity on linear native DNA; and (iii) ATP-independent activity on linear denatured DNA. To determine if these were produced by counterparts of *Eco*ExoVIII, *Eco*ExoV, and *Eco*ExoI, respectively, we fractionated the extract. The bulk of the ATP-independent activity on linear native DNA was lost after Sephadex G-200 chromatography, where it eluted one or two fractions after the activity on linear denatured DNA. The enzyme was very unstable (losing activity after only a few hours) after this elution, and no further studies were undertaken with it. The ATP-dependent activity on linear native DNA and the ATP-independent activity on linear denatured DNA separated on Sephadex G-200 and were each purified additionally by DNA-cellulose chromatography. The successful purification of the ATP-independent activity on native DNA awaits further work.

The ATP-dependent enzyme showed four similarities to *Eco*ExoV: (i) its ATP-dependent activity on linear native DNA; (ii) its production of oligonucleotides as the small-molecular-weight products of digestion; (iii) its inability to degrade cross-linked native DNA; and (iv) its association with a DNA-dependent adenosine triphosphatase activated by cross-linked native DNA. The ATP-dependent activity on linear native DNA was the activity by which *Eco*ExoV was first detected (2, 5, 24), and it was this activity for which the enzyme was named (40).

Where the *Pseudomonas* activity and the *Eco*ExoV differ is that the former does not attack denatured DNA either exo- or endonucleolytically, whereas *Eco*ExoV does. This difference results in a difference in the reaction products found in a limit digest of native DNA by the two enzymes. In the case of *Eco*ExoV, approximately 100% of the substrate is converted to an acid-soluble form (9), whereas in the case of the *Pseudomonas* activity only 56% was rendered acid soluble. The acid-insoluble remainder was presumably in the form of high-molecular-weight single-stranded fragments, which are themselves not substrates for the *Pseudomonas* enzyme.

This is reminiscent of the situation which obtains with the limit digests of other exonucleases active on native and not denatured

DNA, e.g., *Eco*ExoVIII (16), *Lam*ExoVIII (6), and *Eco*ExoIII (28). With these enzymes in excess, the products of the reaction are mononucleotides and high-molecular-weight single-stranded DNA fragments. A mechanism for this type of reaction was proposed by Richardson et al. (28). It is reasonable to suppose that the ATP-dependent *Pseudomonas* nuclease acts in similar fashion, although perhaps not in a unique direction.

ATP-dependent DNA nucleases have been found in a wide variety of bacterial species. It is interesting that these enzymes fall into two groups. The first contains enzymes similar to *Eco*ExoV in that they are active on single-stranded as well as double-stranded DNA and act exo- and endonucleolytically on single-stranded DNA. The enzymes from *Haemophilus influenzae* (7, 8, 22) and *Mycobacterium smegmatis* (29) fall in this group. The second group of ATP-dependent enzymes is similar to the *Pseudomonas* enzyme in that they have no nucleolytic activity on linear denatured DNA. The enzymes from *Micrococcus luteus* (né *Micrococcus lysodeikticus*) (33), *Bacillus subtilis* (23), and *Diplococcus pneumoniae* (34, 35) fall into this group. At present it is not clear whether this distinction results from inactivation of or the separation from group II enzymes of subunits that participate in activity on single-stranded DNA or whether the group II enzymes will be as fundamentally distinct from group I enzymes as type II restriction endonucleases are from the type I (4).

It is a matter of some moment to determine whether or not both groups of ATP-dependent nucleases active on native DNA should be considered to be exonuclease V enzymes. Since the properties these enzymes share are those for which *Eco*ExoV was named, it seems reasonable to consider them part of the same generic class. (There is ample precedent for this; for example, type I and II restriction endonucleases are considered to be EndoR enzymes [31].) Whether this classification will prove to be significant in that the enzymes participate in the same physiological functions remains to be seen. Kushner (15) has shown that temperature sensitivity of the ATP-dependent activity on native DNA of a mutant *Eco*ExoV is correlated with temperature-sensitive recombination proficiency of the mutant strain. This leads to the hypothesis that the ATP-dependent activity on native DNA is necessary for recombination in *E. coli*. Likewise, mutations inactivating *Hin*ExoV (37) lower recombination frequencies somewhat (18, 37) and can be correlated with the failure of one strand of competent cell DNA

to be broken in the presence of transforming DNA (18). Whether or not both groups of enzymes participate in recombination, or, if they do, whether or not they do so in the same way, is open for further investigation.

The second enzyme purified from *P. aeruginosa* was a nuclease active on linear denatured DNA. Because we did not assess the activity of this enzyme on single-stranded, closed circular DNA, we cannot conclude that the enzyme acts exonucleolytically. As it is, the purified enzyme was shown to be active without addition of Mg^{2+} to the reaction mixture but to be inactive in the presence of ethylenediaminetetraacetate. This indicates a probable requirement for divalent cations either for activity or enzyme stability. The limit digest products are 100% acid soluble and consist mostly of mono- and dinucleotides. This set of properties is reminiscent of the properties of *Eco*ExoI rather than those of *Eco*ExoVII, which has no divalent cation and whose reaction products are mostly oligonucleotides.

From the present data on these two enzymes it would be premature to draw conclusions about the pathways of recombination that occur in *P. aeruginosa*. It would, however, not be surprising to find that they are basically those that occur in *E. coli*.

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