Bacteriophage XP-12-Induced Exonuclease Which Preferentially Hydrolyzes Nicked DNA

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An exonuclease has been partially purified from XP-12-infected *Xanthomonas oryzae* which is not found in uninfected *X. oryzae*. Although both the phage-induced exonuclease and the major host exonucleolytic DNase released 5'-mononucleotides, these enzymes differed in their chromatographic behavior, pH optimum, salt inhibition, and heat sensitivity. These two exonucleases preferred different substrates. Nicked native DNA was the best substrate for the phage-induced enzyme, whereas denatured DNA was the best substrate for the host enzyme. Also, the host enzyme had a significant preference for denatured or nicked, normal cytosine-containing DNA (e.g., *X. oryzae* or T7 DNA) over similarly denatured or nicked 5-methylcytosine-rich DNA (namely, XP-12 DNA), whereas the phage-induced enzyme hydrolyzed both types of DNA equally well.

Bacteriophage XP-12 DNA is unique in having all of its cytosine residues replaced by 5-methylcytosine residues (3, 9). In contrast, the DNA of its host, *Xanthomonas oryzae*, contains the four normal bases and <0.5 mol% 5-methylcytosine. The DNA from *Escherichia coli* T-even phage, which contains (glucosylated) 5-hydroxymethylcytosine, is the only other DNA known in which all the cytosine residues are modified (12). T-even phage encode a number of different DNases (1, 21-24, 29), some of which are able to selectively hydrolyze host, that is, cytosine-containing DNA (1, 21, 22, 24). It was, therefore, of special interest to characterize XP-12-induced DNases to determine whether phage infection results in the synthesis of a new DNase activity which might show a preference, not seen with host DNases, for unmodified DNA or modified DNA.

We have partially purified a DNase activity detected in XP-12-infected *X. oryzae* and not in the uninfected cells. The purification and characterization of this XP-12-induced DNase and its comparison to the major DNase of uninfected host cells are described. This phage-induced enzyme hydrolyzed native or denatured DNA with a preference for native DNA which had previously been nicked with either DNase I or micrococcal nuclease. It hydrolyzed 5-methylcytosine-rich DNA and normal, cytosine-containing DNA equally well, unlike the host exonuclease which had a preference for normal, cytosine-containing DNA.

MATERIALS AND METHODS

Bacteria and phage. Bacteriophage XP-12 was propagated and plated on *X. oryzae* 507 (3). For harvesting, cells were grown in aerators in 30°C in XMS medium (3). At an optical density of 0.6 at 550 nm, they were infected with XP-12 phage with a multiplicity of infection of 10 to 20. Infected cells were cooled and harvested at 45 to 180 min postinfection and stored at -50°C until use. Bacteriophage T7 was propagated on *Escherichia coli* B by standard techniques (25).

Nucleic acids. DNA was isolated by standard methods (15, 17). Radioactively labeled DNAs were prepared by labeling bacteria or phage-infected bacteria with [³H]uracil (for *X. oryzae* and XP-12 DNAs) or [³H]thymidine (for T7 DNA). Specific activities of the DNAs ranged from 4,500 to 15,000 cpm/nmol. All XP-12, *X. oryzae* and T7 DNA samples were lightly sheared by passage four times through a 27-gauge needle.

For limited predigestion of 0.4 to 0.9 μmol of [³H]DNA per ml, we used DNase I at 0.19 or 0.38 U/ml for 20 min at 20°C (28) to give 0.5 or 1.2% acid solubility or at 1.2×10^{-3} to 3.6×10^{-2} U/ml for 1 h at 37°C to give 6.2 to 10.6% acid solubility. Considerably more DNase I was necessary for digesting XP-12 DNA because, compared with DNAs of normal base composition, it was relatively resistant to DNase I (K. Ehrlich and M. Ehrlich, unpublished data). Reactions were stopped by heating at 65°C for 15 min. T7 DNA (at 0.4 μmol/ml) was digested to 1.3 or 6.3% acid solubility with 1.3×10^{-3} or 0.14 U of micrococcal nuclease per ml for 20 to 40 min, respectively, at 20°C (18) and then extracted and precipitated (3). Some of these digests were then heat denatured and treated with 0.25 U of *E. coli* alkaline phosphatase per ml for 1 h at 37°C; potassium phosphate (pH 7.5) to 2 mM was subsequently added to inhibit the alkaline phosphatase (28) without affecting the exonuclease activity.

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Enzyme assay. The standard assay for XP-12 exonuclease contained 50 mM Tris-hydrochloride, 10 mM MgCl₂, 0.1 mM EDTA, 0.5 mg bovine serum albumin per ml (pH 7.4 at 37°C), and 2.3 nmol (nucleotide residues) of DNA in 150 μ l. For the assays done during purification, the substrate was native *X. oryzae* [³H]DNA because it was a good substrate for XP-12 exonuclease and a poor one for the host exonuclease. However, for characterization of the purified XP-12 exonuclease, heat-denatured XP-12 [³H]DNA was used because it was a better substrate for XP-12 exonuclease than was nonnicked native DNA. The assays were incubated for 1 h at 37°C, and acid-soluble radioactivity was assayed (5). One unit of enzyme solubilized 1 nmol of nucleotide residue from native *X. oryzae* [³H]DNA in 1 h under standard conditions.

Chromatography of XP-12 exonuclease digestion products. For gel filtration, reaction mixtures were chromatographed directly on a calibrated Bio-Gel P-100 (Bio-Rad) column in 10 mM NaCl-1 mM EDTA-10 mM Tris-hydrochloride (pH 8.0). For thin-layer chromatography, reaction mixtures, in some cases treated with 5'-nucleotidase for 1 h at 37°C, were desalted and concentrated by adsorption to Norit A (14). Then they were chromatographed with internal nonradioactive standards in two dimensions on polyethyleneimine-cellulose plates (19) with water as the solvent in the first dimension and 1 M LiCl in the second dimension. The fast-moving nucleosides were scraped from the plate after chromatography in the first dimension; the remainder of the plate was chromatographed in the second dimension.

Preparation of DNA-free extracts for DNase. *X. oryzae* cells uninfected or infected with XP-12 and harvested 45, 90, 120, or 180 min after infection were suspended in 50 mM Tris-hydrochloride (pH 7.5), 0.1 mM EDTA, 1 mM dithiothreitol, and 60 μ g of phenylmethanesulfonyl fluoride per ml (7) and sonicated. After sonication, the pH was readjusted to 7.5. All steps in the preparation of extracts or in purification were carried out at 0 to 5°C. The extracts were then centrifuged for 15 min at 10,000 $\times$ g, and the supernatants were precipitated with ammonium sulfate to 70% saturation.

The precipitates were collected by centrifugation and dissolved in sufficient 10 mM Tris-hydrochloride (pH 7.5)-0.1 mM EDTA-10% glycerol-2 mM 2-mercaptoethanol to bring the conductivity to 3.95×10^4 μ mho, or equal to that of 0.25 M KCl. These were then stirred for 2 h with prewashed DEAE-cellulose (2) which had been equilibrated with the above buffer plus 0.25 M KCl. The resin, essentially all of the DNA, and most of the RNA were removed by centrifugation (40,000 $\times$ g for 20 min and 65,000 $\times$ g for 30 min). The supernatants were dialyzed overnight against several changes of 10 mM potassium phosphate (pH 7.5)-2 mM 2-mercaptoethanol-0.1 mM EDTA-10% glycerol (buffer A).

RESULTS

Purification of XP-12 exonuclease. Figure 1 shows that DNase activity capable of solubilizing native *X. oryzae* or XP-12 DNA was low in DNA-free crude extracts of uninfected cells

FIG. 1. DNase activity of DNA-free extracts from uninfected and XP-12-infected *X. oryzae*. As described in the text, extracts were prepared from uninfected cells and from XP-12-infected cells harvested 45, 90, 120, or 180 min after infection, and 25- to 50- μ l portions were assayed in the standard assay for DNase activity on denatured DNA (A), native DNA (B), or DNase I-nicked DNA (C). Approximately 0.3 mg of protein was used per assay. Symbols: ●, *X. oryzae* DNA; ○, XP-12 DNA.

but began to increase in extracts from cells harvested at 90 min postinfection. The latent period of *X. oryzae* grown under the conditions used in these experiments was approximately 4 h.

For purification of XP-12 exonuclease, a DNA-free extract was prepared from 11 g of XP-12-infected *X. oryzae* harvested 180 min after infection. It was loaded onto a DEAE-cellulose equilibrated with buffer A and eluted with a linear gradient of 0 to 0.75 M KCl in buffer A. Figures 2 and 3 show that induced exonuclease activity could be distinguished from the exonuclease activity observed in uninfected *X. oryzae* (Farber and Ehrlich, unpublished data) by its greater affinity for DEAE-cellulose, much greater ability to digest double-stranded DNA, and significantly improved ability to hydrolyze denatured XP-12 DNA relative to denatured *X. oryzae* DNA.

For this chromatograph and for the phospho-

FIG. 2. DEAE-cellulose chromatography of DNA-free extract from XP-12-infected *X. oryzae* 180 min postinfection. Preparation of DNA-free extracts for chromatography and DNase assay were as described in the text. The samples were chromatographed on a 1.6- by 45-cm column as described in the text, and 13-ml fractions were collected. Symbols: ■, denatured *X. oryzae* DNA; □, denatured XP-12 DNA; ●, native *X. oryzae* DNA; ○, native XP-12 DNA; △, KCl concentration.

FIG. 3. DEAE-cellulose chromatography of DNA-free extract from uninfected *X. oryzae*. Chromatography and assays were as in Fig. 2. Symbols: ■, denatured *X. oryzae* DNA; □, denatured XP-12 DNA; ●, native *X. oryzae* DNA; ○, native XP-12 DNA; △, KCl concentration.

cellulose chromatograph, XP-12 DNA and *X. oryzae* DNA pretreated with DNase I to 9.2 and 6.2% acid solubility, respectively, were also used as substrates. Peaks of enzyme activity on these substrates were seen only in the XP-12 exonuclease and host exonuclease peaks.

After precipitation of the pooled XP-12

DNase, DEAE-cellulose fractions with 70% saturated ammonium sulfate and dissolution in and dialysis against buffer A, the sample was applied to a 0.9- by 15-cm phosphocellulose column equilibrated with that buffer. All of the XP-12 exonuclease activity appeared in the flow-through and rinse fractions. These were precipitated with 70% saturated ammonium sulfate and resuspended in 50 mM Tris-hydrochloride (pH 7.5)-20 mM KCl-0.1 mM EDTA-10% glycerol-2 mM 2-mercaptoethanol (buffer B). After dialysis against buffer B, the sample was chromatographed on a DEAE-Sephadex A-25 column with a linear gradient of 0.02 to 1 M KCl in buffer B. XP-12 exonuclease eluted from the column at 0.19 to 0.25 M KCl. Pooled peak fractions were dialyzed against buffer A with additional 50% glycerol and 50 mM KCl.

An aliquot of the XP-12 exonuclease was further purified on a hydroxyapatite column with a linear gradient of 0.01 to 0.7 M potassium phosphate (pH 7.5) in buffer A. XP-12 exonuclease eluted from the column at approximately 0.032 M potassium phosphate. The active fractions were stored at -20°C after addition of glycerol to 50% and of acidified, boiled, and neutralized (10) bovine serum albumin to 1 mg/ml. The DEAE-Sephadex and hydroxyapatite fractions proved to be stable at -20°C and indistinguishable in their enzymatic properties.

The partial purification of XP-12 exonuclease is summarized in Table 1. The increase in enzyme activity in step II is probably due to removal of a nonspecific inhibitor. This enzyme is much less stable than the host exonuclease and is obtained in much lower yield. The DNase activity in the first DEAE-cellulose peak (Fig. 2), which resembled the host exonuclease, was also partially purified and was indistinguishable from the exonuclease of uninfected cells. Furthermore, there was no decrease in its yield or in the yield of two host restriction endonucleases (R. Wang, J. Shedlarski, M. Farber, D. Kuebbing, and M. Ehrlich, *Biochim. Biophys. Acta*,

TABLE 1. Purification of XP-12 endonuclease

Fraction	Total U	Sp act (U/mg of protein)
I Crude low-speed supernatant	144	0.0886
II DNA-free extract	434	0.474
III DEAE-cellulose eluate	724	7.01
IV Phosphocellulose flow through	153	6.22
V DEAE-Sephadex A-25 eluate	67.7	10.4
VI Hydroxyapatite eluate ^a	5.03	138

^a Only approximately one sixth of the A-25 eluate was used for hydroxyapatite chromatography.

in press) at 180 min postinfection.

No endonuclease activity was detected in the DNA-free extract or in the DEAE-cellulose chromatography fractions when *X. oryzae*, XP-12, or T7 DNAs were used as substrates and the molecular weight of the DNAs was monitored by electrophoresis in 1% agarose gels under standard conditions (6). Also, no endonuclease activity was seen when up to 75 μ g of protein in the DNA-free extracts was assayed in the DNA-containing polyacrylamide gel electrophoresis system of Rosenthal and Lacks (20) with either calf thymus DNA or XP-12 DNA as the substrate under conditions in which 0.1 ng of DNase I was easily detectable.

Properties of XP-12 exonuclease. XP-12 exonuclease had an absolute requirement for Mg^{2+} , which could not be replaced by Ca^{2+} or Mn^{2+} . The presence of a sulfhydryl reagent enhanced activity, and *N*-ethylmaleimide partially inhibited activity. Nucleoside monophosphates at relatively high concentrations (100 μ M) resulted in less than 15% inhibition of enzyme activity, and the addition of ATP to the reaction mixture gave no significant change in the enzyme activity. The omission of bovine serum albumin from the reaction mixture resulted in a 70% decrease in activity.

That the partially purified phage-induced nuclease is an exonuclease is indicated by the kinetics of hydrolysis, the characterization of essentially all of the low-molecular-weight reaction products as mononucleotides, and the chromatographic behavior of partially digested DNA. The time course of the reaction, as monitored by the appearance of acid-soluble products, was linear at the start of the reaction, with no detected lag period (data not shown). At least 75% of denatured DNA and 40% of native DNA could be digested.

To examine the low-molecular-weight products of the reaction, XP-12 [3H]DNA was incubated with XP-12 exonuclease until 23% of the label became acid soluble. The digestion products were then examined by thin-layer chromatography with or without prior treatment with 5'-nucleotidase, which was shown not to contain detectable 3'-nucleotidase activity. More than 96% of the low-molecular-weight, radioactively labeled material was converted to nucleosides after incubation with XP-12 DNase followed by 5'-nucleotidase treatment. Without incubation with 5'-nucleotidase, 92% of the low-molecular-weight products chromatographed as mononucleotides.

When denatured XP-12 [3H]DNA was digested with XP-12 exonuclease to 4 or 34% acid solubility and then chromatographed on a Bio-Gel P-100 column, only a major peak in the

excluded volume and a minor peak coeluting with mononucleotides were observed. Analogous results were obtained on native XP-12 [3H]DNA digested to 1.0 or 4.5% acid solubility.

XP-12 exonuclease differed significantly from the host exonuclease in many of its properties. Whereas XP-12 exonuclease showed a rather sharp pH optimum at approximately pH 7.0 (phosphate buffer), *X. oryzae* exonuclease displayed a broad optimum range of approximately pH 7.0 to 7.5. The XP-12 exonuclease-catalyzed hydrolysis of DNA was inhibited 18% by 50 mM KCl and 43% by 100 mM KCl. In contrast, *X. oryzae* exonuclease showed no inhibition with KCl at concentrations of up to 100 mM. Preincubation of XP-12 exonuclease at 37°C in reaction buffer for 30 min or assay at 42°C (instead of 37°C) decreased its activity approximately 70%, whereas incubation of *X. oryzae* exonuclease under the same conditions gave less than a 5% reduction in activity.

Substrate specificity. The relative activity of XP-12 exonuclease and the host exonuclease on a variety of sheared, nicked, or denatured DNA substrates was compared. Table 2 shows that XP-12 exonuclease hydrolyzed nonnicked native DNA considerably more efficiently than did the host enzyme. Also, XP-12 exonuclease did not have the marked preference for *X. oryzae* DNA over XP-12 DNA which was seen for *X. oryzae* exonuclease. Another difference between these exonucleases is that limited micrococcal nuclease predigestion of the DNA stimulated XP-12 exonuclease but inhibited *X. oryzae* exonuclease unless the 3'-phosphate termini were removed by alkaline phosphatase. Predigestion of the DNA with DNase I to 0.5% acid solubilization stimulated XP-12 exonuclease markedly, although only a slight stimulation of *X. oryzae* exonuclease was observed. This limited predigestion apparently resulted mostly in nicking and not in appreciable formation of single-stranded regions since subsequent treatment with the single-strand-specific nuclease S1 (27) resulted in no detectable acid solubilization of these DNA preparations, although it did solubilize more extensively predigested DNA. When the DNA was more extensively predigested by DNase I, the activity of the exonuclease was further increased, although the rate of increase was much less than the extent of predigestion. Denaturation of DNase I- or micrococcal nuclease-nicked DNA resulted either in only a slight increase or in a decrease in the ability of the exonuclease to hydrolyze this DNA.

DISCUSSION

Infection of *X. oryzae* with phage XP-12 has been shown to induce the production of a nu-

TABLE 2. Hydrolysis of various predigested or denatured DNAs by XP-12 exonuclease and *X. oryzae* exonuclease^a

DNA	Enzyme used for pretreatment of DNA	Acid solubilization of predigested DNA (%)	Denaturation of DNA	Enzyme activity (nmol of residues solubilized per h)	
				XP-12 exonuclease	<i>X. oryzae</i> exonuclease
<i>X. oryzae</i>	None		—	0.05	0.08
	None		+	0.17	0.68
	DNase I	0.5	—	0.21	0.11
	DNase I	1.2	—	0.30	0.25
	DNase I	6.2	—	0.71	0.40
XP-12	None		—	0.05	0.01
	None		+	0.13	0.18
	DNase I	9.2	—	0.67	0.20
T7	None		—	0.14	0.13
	None		+	0.16	0.75
	DNase I	10.6	—	0.47	0.48
	DNase I	10.6	+	0.53	1.38
	DNase I, phosphatase	10.6	+	0.58	1.68
	Micrococcal nuclease	6.3	—	0.86	0.05
	Micrococcal nuclease	6.3	+	0.51	0.67
	Micrococcal nuclease, phosphatase	6.3	+	0.47	0.99

^a Assays were performed under standard conditions with 2.3 nmol of DNA and 0.7 U of *X. oryzae* exonuclease or 0.05 U of XP-12 exonuclease. The pretreatment of the DNAs with DNase I or micrococcal nuclease with or without additional treatment with alkaline phosphatase is described in the text.

clease activity not seen in uninfected host cells. This nuclease is an exonuclease which released 5'-mononucleotides; it can hydrolyze both native and denatured DNA. It showed no preference for *X. oryzae* DNA or T7 DNA, normal, cytosine-containing DNAs (9, 13), over XP-12 DNA, a uniquely 5-methylcytosine-rich DNA (3, 9), or vice versa (Table 2). In contrast, the main host exonucleolytic DNase showed approximately a threefold preference for the normal, cytosine-containing DNAs (unpublished data).

The properties of XP-12 exonuclease make it readily distinguishable from *X. oryzae* exonuclease, the major detected host DNase (apart from two restriction endonucleases *XorI* and *XorII*; R. Wang, J. Shedlarski, M. Farber, D. Kuebbing, and M. Ehrlich, *Biochim. Biophys. Acta*, in press). Although both enzymes are exonucleases which release 5'-mononucleotides, they are clearly different by chromatographic behavior (Fig. 2 and 3), heat sensitivity, effect of salt concentration and pH on the reaction rate, inhibition by phosphorylated termini (Table 2), and substrate preference. The best substrate for *X. oryzae* exonuclease was denatured DNA, whereas the best substrate for XP-12 exonuclease was nicked native DNA (Table 2 and unpublished data). Relative to denatured DNA, native DNA was a better substrate for XP-12 exonuclease than for *X. oryzae* exonuclease (Table 2).

Upon infection of the host with XP-12, the

host exonuclease is neither inactivated nor detectably modified. The additional phage-induced exonuclease may provide a capability for digesting nicked XP-12 DNA that is not present in sufficient quantity in the uninfected host. This phage-induced enzyme may be necessary for some aspect of phage DNA metabolism, such as DNA recombination, as has been demonstrated for T7 exonuclease (8, 11, 16, 26). Such a function would be consistent with the appearance of relatively large amounts of XP-12 exonuclease activity at the peak period of phage DNA synthesis (4).

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