SUSCEPTIBILITY OF NON-THYMINE CONTAINING DNA TO FOUR BACTERIAL RESTRICTION ENDONUCLEASES

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1. Introduction

A number of bacterial restriction endonucleases have recently been isolated and their specificities toward DNA characterized (see Review [1–3]. Some of the restriction endonucleases, such as EcoR1 and HpaI, specifically cleave unmodified double-stranded DNA [3]. The sequence of nucleotides cleaved by these restriction endonucleases have 180° rotational symmetry [1–4]. It has been shown [5] that EcoR1 endonuclease introduces cleavage in DNA at

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      ↓
5' . . . G - A - A - T - T - C . . . 3'
3' . . . C - T - T - A - A - G . . . 5'
                  ↑
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On the other hand, HpaI endonuclease [6] introduces cleavage in DNA at

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          ↓
5' . . . G - T - T - A - A - C . . . 3'
3' . . . C - A - A - T - T - G . . . 5'
              ↑
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Recently, it has been established [7] that HindIII endonuclease digests double-stranded DNA at

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      ↓
5' . . . N - A - A - G - C - T - T - N . . . 3'
3' . . . N - T - T - C - G - A - A - N . . . 5'
                  ↑
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The cleavage products of EcoR1 and HindIII have cohesive termini, while the products of HpaI digestion do not.

Since targets for these three enzymes contain A-T base pairs, it was of interest to determine whether these endonucleases attack DNA molecules not containing thymine. There are many *Bacillus subtilis* phages which contain hydroxymethyluracil instead of thymine in the DNA molecule [8]. In the present communication, we will show that phage SPO1 DNA [9], one of the hydroxymethyluracil-containing *B. subtilis* phage DNAs, is sensitive to EcoR1 and HindIII endonucleases but is resistant to HpaI endonuclease. It was also found that SPO1 DNA is sensitive to HpaII endonuclease, which cleaves DNA at the following nucleotide sequence (6):

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      ↓
5' . . . N - C - C - G - G - N . . . 3'
3' . . . N - G - G - C - C - N . . . 5'
              ↑
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2. Materials and methods

2.1. *Preparation of phage DNA*

Bacteriophage SPO1 was prepared as described previously [10]. Purified phage particles (5×10^{11} pfu/ml) were treated with SDS (2%) and heated at 60°C for 2 min. After cooling to 40°C, proteinase K (E. Merck) was added to a final concentration of 200 μg/ml and incubated for 60 min. DNA was then extracted twice with phenol, precipitated with ethanol, and dissolved in 0.1 × SSC (SSC is 0.15 M NaCl and

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0.015 M sodium citrate) containing 0.1 mM (ethylenediamine) tetraacetate (EDTA). ϕ29 DNA and λDNA were prepared as described previously [11].

2.2. *Hydrolysis of DNAs by endonucleases*

DNA (20–25 μg) in 0.1 M Tris–HCl (pH 7.5), 0.01 M $MgCl_2$ and 0.05 M NaCl was incubated with EcoR1 (2 μl) or HindIII (5 μl) endonuclease (Miles Laboratories). After incubation for three hours at 35°C, the hydrolysis was terminated by the addition of EDTA to 10 mM and heating at 60°C for 5 min.

Digestion of DNA with HpaI or HpaII endonucleases was performed according to the method of Sharp, Sugden and Sambrook [12]. Endonuclease HpaI was prepared from *Haemophilus parainfluenzae* according to Sharp et al. [12]. Endonuclease HpaII was purchased from Miles Laboratories.

2.3. *Agarose gel electrophoresis*

Agarose gels (1% w/v) were prepared by suspending the agarose (MCI Biomedical, Maine) in electrophoresis buffer (Tris-borate-EDTA, pH 8.3) and boiling [13]. The homogeneous solution was poured into the slab gel apparatus (12 × 16 × 0.3 cm) after cooling to 60°C. The samples (20 μl) containing untreated or endonuclease-treated DNA were adjusted to 8% glycerol and 0.01% bromophenol blue and heated at 60°C for 5 min before loading onto the gel. Electrophoresis was carried out at a constant current of 30 mA at room temperature. The gels were stained in

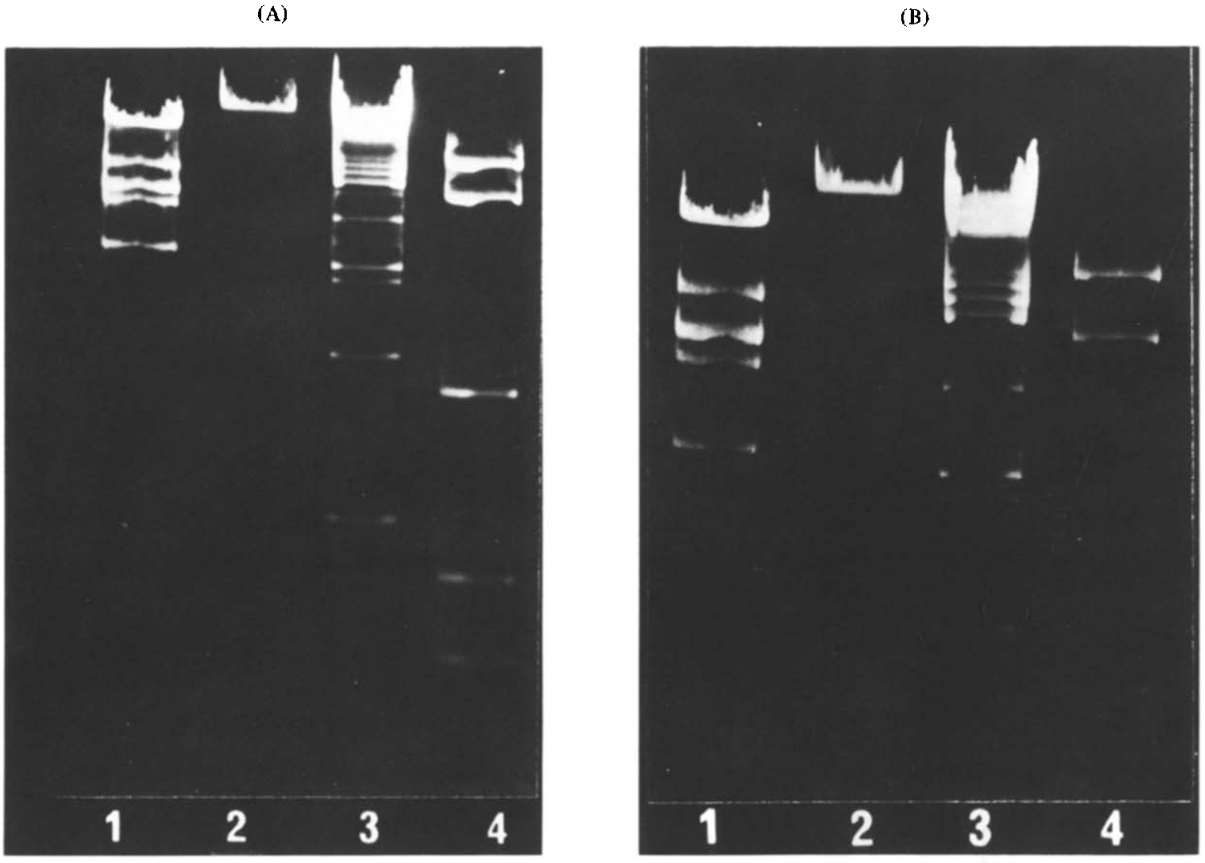

Fig.1. Agarose gel electrophoresis of SPO1 DNA and SPO1 DNA digested by EcoR1 endonuclease. Digested DNA (0.5–0.8 μg) was adjusted to 8% glycerol and 0.01% bromophenol blue and heated for 2 min at 60°C. Samples in 20 μl, were applied to 1% agarose slab gel (12 × 16 × 0.3 cm). Electrophoresis was carried out at a constant current of 30 mA. The gel was stained with ethidium bromide and photographed as described in Materials and methods. Fragments of ϕ29 and λDNAs generated by EcoR1 digestion were used as standard markers [11, 15]. (1) λDNA digested with EcoR1; (2) SPO1 DNA; (3) SPO1 DNA digested with EcoR1; (4) ϕ29 DNA digested with EcoR1. (A) 4 hr electrophoresis. (B) 6 hr electrophoresis.

electrophoresis buffer with 0.5 μg of ethidium bromide/ml for 30 min and photographed under a short wave length ultraviolet light, using Polaroid type high speed #57 film and a Kodak #23A red filter.

3. Results and discussion

The results of electrophoresis through agarose gels of the products obtained after digestion of SPO1 DNA, φ29 and λDNA with EcoR1 are shown in fig. 1, A and B. It is evident that EcoR1 cleaves SPO1 DNA into more than 15 fragments. It has been shown previously that this enzyme cuts φ29 DNA and λDNA into 5 [11, 14] and 6 fragments [15], respectively. Since many bands of the SPO1 DNA fragments generated by EcoR1 appear to be overlapping, it is difficult to determine the exact number and molecular weights.

Fig. 2 shows the results of agarose gel electrophoresis of the products obtained after incubation of SPO1 DNA with various restriction endonucleases. φ29 DNA fragments generated by EcoR1 and HpaI endonucleases are also shown in this figure for references. Clearly, HindIII and HpaII, as well as EcoR1, cleave SPO1 DNA into many fragments. However, HpaI endonuclease appears to be inactive toward SPO1 DNA, although the same enzyme digests φ29 DNA [11]. Similar results were obtained with DNAs of φe and φ82, which are also hydroxymethyluracil-containing *B. subtilis* phages, suggesting that these phenomena are not unique for SPO1 DNA.

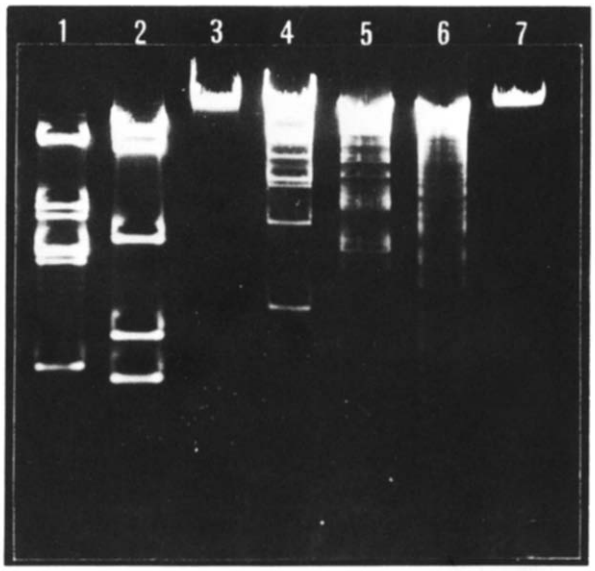

Fig.2. Agarose gel electrophoresis of SPO1 DNA digested by EcoR1, HindIII, HpaII and HpaI endonucleases. The conditions of agarose gel electrophoresis were as described in fig.1. Electrophoresis was carried out for 4 hr at room temperature. (1) φ29 DNA digested with HpaI; (2) φ29 DNA digested with EcoR1; (3) SPO1 DNA treated with HpaI; (4) SPO1 DNA digested with EcoR1; (5) SPO1 DNA digested with HindIII; (6) SPO1 DNA digested with HpaII; (7) SPO1 DNA undigested.

These results can be interpreted in terms of the cleavage sites of these endonucleases: EcoR1 cleaves phosphodiester linkages between guanidylic and adenylic acid residues in both strands and HindIII cuts phosphodiester bonds between two adenylic acid residues. On the other hand, HpaI hydrolyses phosphodiester linkages between thymidylic and adenylic acid residues. Resistance of SPO1 DNA to HpaI cleavage suggests that the presence of thymidylic acid residues adjacent to adenylic acid residues is absolutely essential for enzyme activity. Although two endonucleases, EcoR1 and HindIII, can digest SPO1 DNA, preliminary studies of the kinetics of enzyme digestion suggest that the rate of hydrolysis of SPO1 DNA is considerably slower than that of thymine-containing DNAs, such as φ29 DNA. This may indicate that the maximal activity of these two enzymes is also dependent on the presence of complementary thymidine residues.

Phages containing DNA in which hydroxymethyluracil is substituted for thymine are presently being used to study phage development and phage gene expression in sporulating cells in *B. subtilis* [16–22]. The availability of specific DNA fragments should be of great use in further analysis of these phage systems; for example, comparing the homology of hydroxymethyluracil containing DNAs and isolation of DNA fragments containing the promotor regions of phage modified and unmodified RNA polymerases.

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