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HYDROXYAPATITE CHROMATOGRAPHY OF *BACILLUS SUBTILIS* NUCLEIC ACIDS

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ABSTRACT

Chromatography of *Bacillus subtilis* nucleic acids on hydroxyapatite has been examined. Newly synthesized nucleic acids were labelled during spore germination by P^{32} incorporation. Nucleic acids were eluted from hydroxyapatite columns by passing an increasing linear phosphate molarity gradient through the columns. Three peaks were obtained. These peaks (1,2, and 3) were examined in detail using a variety of techniques. From these data it may be concluded that peak 3 is newly synthesized native *B. subtilis* DNA, and peak 2 is newly synthesized RNA. Peak 1 appears to consist of low molecular weight DNA fragments of unusually heavy buoyant density in CsCl. It is suggested that peak 1 fragments come from the replicating points of the DNA molecule near the origin.

I. INTRODUCTION

The separation of nucleic acids by hydroxyapatite (HA) chromatography has been reported by Bernardi et al.,[1,2] Chevallier et al.,[3] Miyazawa and Thomas,[4] Main and Cole,[5] and others. In general this technique is a rapid, convenient method of separating DNA from RNA as well as single-stranded DNA from double-stranded DNA. The application of this technique to the separation of Bacillus subtilis nucleic acids constitutes the subject of this communication.

In brief, the separation and identification of nucleic acids synthesized during spore germination have been examined. An unusual DNA component has been isolated. Several properties of this DNA will be presented.

II. MATERIALS AND METHODS

Bacillus subtilis strain 168 (indole⁻) and strain W-23 (streptomycin-resistant) were used throughout the studies. Spore preparations were suspended in water, heated at 65 C for 2 hours, and stored at 5 C. Outgrowth experiments were performed with SCM media[6] supplemented with P^{32} (carrier-free, obtained from Oak Ridge National Laboratories) according to the following design. Spores were inoculated to a Klett reading of 40 to 50 (No. 42 filter) in SCM media plus P^{32}. The cultures (usually 10 ml total volume) were incubated at 37 C with aeration. Samples were taken at the times indicated in Figure 1, heated at 60 C for 10 minutes, harvested by centrifugation and washed twice with 0.001 M sodium phosphate buffer, pH 6.8. The cells, taken up in the same buffer, were incubated with 50 to 100 µg/ml lysozyme (crystallized egg white lysozyme, Armour Pharmaceutical Co., Kankakee, Ill.) at 37 C for 1 hour. Lysis was completed by addition of sodium lauryl sulfate. The lysates were extracted with water-saturated phenol ("Gilt Label," Mallinckrodt Chemical Works, N.Y.) in the cold for 30 minutes. Phenol and aqueous phases were separated by centrifugation. Residual phenol was removed by dialysis of the aqueous phase against 0.001 M phosphate buffer in the cold. An appropriate dilution of the extract in 0.001 M phosphate buffer was loaded on the hydroxyapatite column.

Column procedures were essentially as reported by Bernardi.[1] Elution was performed by passing a linear gradient of increasing phosphate molarity through the column. P^{32} was measured with a Nuclear Chicago liquid scintillation system.

A large number of experiments involving diverse techniques are summarized in Table 1. The methods used in these experiments are included in the table.

III. RESULTS AND DISCUSSION

Figure 1 depicts typical elution profiles obtained from outgrowth experiments using B. subtilis spores. Fifteen-, 30-, and 60-minute samples are shown. The peaks are designated: peak 1, the earliest eluting material (about fraction no. 6), which corresponds to a phosphate molarity of about 0.06 M; peak 2, the second eluting peak (about fraction no. 17), corresponding to about 0.22 M phosphate; peak 3, the trailing shoulder of peak 2 (fractions 21, 22), which elutes at about 0.28 M phosphate. (This is the region in which native B. subtilis DNA prepared by Marmur's method[7] is found to elute.) Similar elution profiles have been obtained from log phase cells of strain 168 as well as from spore outgrowth studies of strain W-23.

Table 1 presents the results of a series of experiments designed to elucidate the composition of peaks 1, 2 and 3 obtained from 1-hour outgrowth of strain 168 spores. The following conclusions may be drawn from the data presented in the table. Peak 3 consists of native double-stranded B. subtilis DNA. Peak 2 consists largely of newly synthesized RNA. A small amount of DNA may be present in peak 2. Peak 1 appears to consist largely of DNA of unusual density.

The finding that extremely low molecular weight DNA fragments present in bacteriophage SP-10 DNA preparations elute from HA at about 0.06 M phosphate,[8] and the report by Main and Cole[6] that polynucleotides elute from calcium phosphate columns at similar phosphate molarity, suggested the possibility that peak 1 may also consist of low molecular weight fragments. The following properties of peak 1 are compatible with this suggestion: i) peak 1 material forms a broad heterodisperse band in CaCl density gradients; ii) digestion with DNAse does not alter the elution position of peak 1 from HA; iii) peak 1 material does not quantitatively bind to nitrocellulose membranes after melting and ice quenching;[9] iv) no homology can be detected between peak 1 and B. subtilis DNA or RNA by standard annealing techniques.

The unusual buoyant density of peak 1 in CsCl remains unexplained. Several suggestions may be offered, however. i) Peak 1 may contain hydroxymethyl uracil residues as are found in several B. subtilis bacteriophage DNAs.[10] ii) Peak 1 may consist of G-C rich fragments of DNA. Hanawalt and Ray[11] have shown that replicative points of DNA are extremely shear-sensitive. Furthermore, O'Sullivan et al.[12] have shown that the segment of the B. subtilis DNA molecule near the point of initiation of replication has an unusually heavy buoyant density and is likely to have a G-C rich region. It is quite possible therefore, that peak 1 material consists of G-C rich DNA fragments that were produced by shearing of the replicative points of the DNA molecule. iii) Peak 1 fragments may contain small amounts of RNA. It is hoped that further studies will resolve these possibilities.

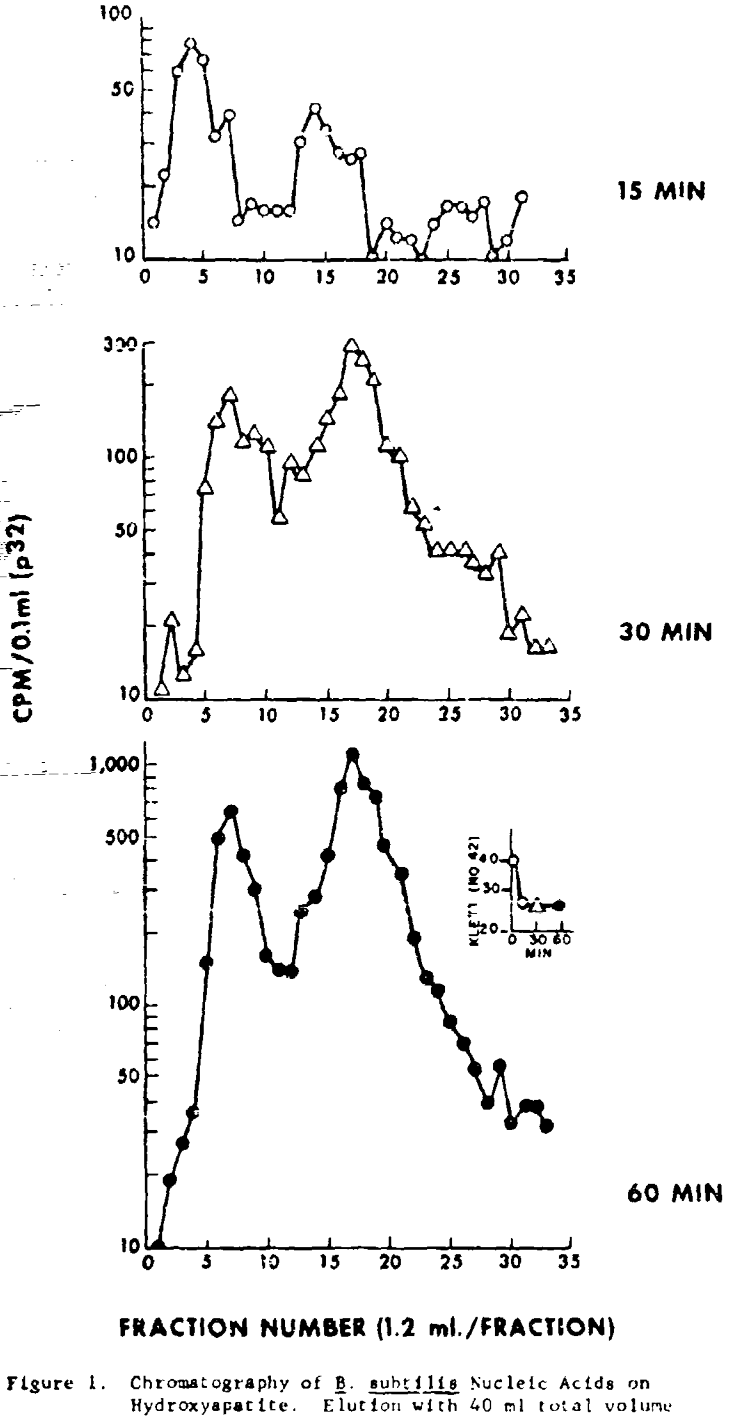

Figure 1. Chromatography of B. subtilis Nucleic Acids on Hydroxyapatite. Elution with 40 ml total volume linear phosphate molarity gradient (0.01 M to 0.5 M). Peak 1, about fraction 6; Peak 2, about fraction 17; Peak 3, about fraction 21, 22.

TABLE 1. SUMMARY OF EXPERIMENTS DESIGNED TO ELUCIDATE THE COMPOSITION OF PEAKS 1, 2, AND 3 INDICATED IN FIGURE 1[a]

Design	Materials Tested	Results	Comments
1. Phenol re-extraction, then chromatographed on HA	Mixture 1,2,3	1,2,3	No phenol-soluble material affecting profiles
2. Rechromatography of lesser amounts on HA	Mixture 1,2	1,2	Column not overloaded
3. Dialysis against 0.001 M PB	Mixture 1,2,3	No P^{32} outside tubing up to 72 hr	No dialyzable P^{32} components
4. RNAse (Worthington) 50 μg/ml, 37 C, 1 hr; dialysis 4 C vs. 0.001 M PB, then chromatographed on HA	Mixture 1,2,3	1,3	2 is RNAse-sensitive
5. DNAse (Worthington) same as RNAse with addition of 0.01 M Mg^{++}	Mixture 1,2,3	1,2 (3 not resolved)	1,2 not DNAse-sensitive
6. Alkaline hydrolysis, 0.5 M KOH, 24 hr at rt; $HClO_4$ precipitated. Results show % original counts solubilized	1 2 3	30% 80% 20%	2 is largely hydrolyzed compatible with RNA
7. Binding to nitrocellulose membranes. Method of Nygaard & Hall.[13] Results show % original counts bound after:			
A. Heated 95 C 15 min in 2X SSC followed by ice quench	1 2 3	26% 12% 98%	3 is DNA, some DNA possible in 1 and 2
B. Heated as in A. in presence of 168 DNA. Incubated at 70 C 2 hr. Brought to rt overnight	1 2	3 2	2 anneals with 168 DNA
C. Heated as in A.; annealed with no additions as in B	1 2	2 5	Some hybrids formed in 2 indicating small amount of DNA present in 2

8. Thermal denaturation by heating 95 C. 15 min in 2X SSC followed by:			
A. Annealing as in 7B. Then rechromatographed on HA	1,2	1,2	No homology detected
	1, 168 DNA	1	No homology detected
	1, salmon sperm DNA	1	No effect of heterologous DNA
	2	2	No self-annealing
	2, 168 DNA	1 broad	Shift due to DNA/RNA hybrid formation
B. Ice quenched, then rechromatographed on HA	2, salmon sperm DNA	2	No effect of heterologous DNA
	2	2	No evidence of strand separation
	168 DNA (peak 3)	2	Shift due to single-strand DNA production
C. Ice quenched followed by:			
DNAse	1	1	Nuclease sensitivity not produced by strand separation techniques.
RNAse	1	1	
9. Buoyant density in CsCl 35,000 rpm, 72 hr, 25 C, SW-39 rotor of Spinco Model L. Density determined by refractive index using Bausch & Lomb Abbe-3L	1	Broad heterodisperse band[b] avg density 1.743 g/cc	Probably low molecular weight DNA of high G-C
	3	Sharp band of 1.703 g/cc	Native *B. subtilis* DNA
	2	Sediments to bottom of tube ~1.780 g/cc	RNA

a. Abbreviations used in table: HA = hydroxyapatite, PB = sodium phosphate buffer pH 6.8, G-C = guanine-cytosine, rt = room temperature, DNA = deoxyribonucleic acid, RNA = ribonucleic acid, DNAse = deoxyribonuclease, RNAse = ribonuclease, SSC = 0.15 M NaCl, & 0.015 M sodium citrate, 2X SSC = twice the concentration of SSC, 168 DNA = DNA from log phase cells of strain 168 prepared by Marmur's method,[7] 1,2,3 = references to peaks eluting from HA as described in the text.

b. Distribution in CsCl unaffected by RNAse digestion according to method of Hayashi, Hayashi and Spiegelman.[14]

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