FRACTIONATION OF NUCLEIC ACIDS FROM DORMANT AND GERMINATED AZOTOBACTER CYSTS*

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Summary.

Chromatography of nucleic acid extracts of Azotobacter vinelandii cysts and of phased germinated cysts was performed using methylated albumin-kieselguhr(MAK) columns. After 4 hours germination, DNA and ribosomal RNA peaks are seen to elute at higher NaCl concentrations than those of dormant cysts. The elution profile of transfer RNA did not change during cyst germination. Evidence is presented which suggests that shifts in elution profiles are due to changes in nucleic acid configuration during the process of germination. This evidence may indicate an additional mechanism for cyst survival in a deleterious environment.

The normal Azotobacter vegetative cell is a rather long, thick encapsulated gram-negative rod with a thin cell wall and a fairly homogeneous cytoplasm. As described by Wyss, Neumann and Socolofsky (1961), through a four stage process the vegetative cell is transformed over a period of 4 to 5 days into a spherical resting cell termed a cyst. The cyst consists of a compact, spherical replica of the vegetative cell, the central body, which is surrounded by a dense double-layered coat structure. Germination of the cyst results in a normal vegetative cell after 6 to 8 hours in a growth medium.

The physical and resistant properties of cysts have been well documented (Socolofsky and Wyss, 1961; Socolofsky and Wyss, 1962). Parker and Socolofsky (1966) have reported on various properties of the isolated central body of the cyst. Although there are several reports concerning composition of cysts (Olson and Wyss, 1967; Olson and Wyss, 1968; Lin and Sadoff, 1968), there is no documented research on the nucleic acids of the cyst. The purpose of the present investigation was to monitor the nucleic acids during the cyst germination process. Evidence is presented which indicates qualitative differences between the nucleic acids of dormant and germinating cysts

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METHODS

Organism. A. vinelandii, ATCC 12837, was the organism used in these studies for the production of vegetative cells and cysts. Cells were grown in Burk's nitrogen-free salts broth (Wilson and Knight, 1952) at 33 C with 0.5% sucrose as the carbon source. Cysts were obtained by growth at 33 C on Burk's agar medium with 0.2% n-butanol as the carbon source. Cysts were harvested after 9 to 10 days; vegetative cells were collected at 18 hours.

Phased Germination. Phased germination of washed dormant cyst suspensions was obtained using a modification of the temperature shift methods reported by Zaitseva and Vedenina (1965) and Lin and Wyss (1965). Approximately 1 x 10^7 cysts per ml were placed in sterile Burk's broth without sucrose and held at 1 C for 50 minutes followed by 33 C for 15 minutes. Sucrose was then added and the culture was placed on a rotary shaker at 33 C. At zero time and at half-hour intervals thereafter, samples of the culture were removed and optical density was recorded with a Klett-Summerson colorimeter with no.42 filter. In accordance with the report of Smith and Wyss (1969), viable cell counts were made by the dilution method with Burk's broth as diluent after 24 hours growth on Burk's agar with 0.5% sucrose.

Nucleic Acid Isolation. Total nucleic acids were extracted at 0,2, 4,6, and 7.5 hours from phased germinating cyst cultures. To stop nucleic acid synthesis at the various time intervals during germination, suspensions were poured over frozen Burk's salts medium, collected by centrifugation and washed at 4 C in 25mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4). The pellet was then suspended in 5mM ethylenediaminetetraacetic acid(EDTA) and carried through two freeze-thaw cycles to ensure maximum rupture of the cyst coat structure. Following centrifugation, the pellet of released central bodies was then resuspended and washed twice in 0.1M EDTA + 0.15M NaCl at pH 8.0. The central bodies were then suspended in 40 ml EDTA-NaCl buffer plus 4 ml 0.2M Tris with 1 mg per ml lysozyme. After mild shaking for 15 minutes, complete lysis of the central body suspension was obtained by addition of sodium lauryl sulfate to a final concentration of 2.0%. After mild shaking, the nucleic acids were extracted with equal volumes of liquefied phenol according to the method of Gierer and Schramm (1956). After the third precipitation with 3 volumes of 95% ethyl alcohol, the final precipitate was dissolved in 50mM sodium phosphate buffer (pH 6.3), containing 0.1M

NaCl. In all experiments approximately 1 mg nucleic acid (a total of 25 optical density units at 255mμ) was applied to 2.0 by 7.5 cm MAK columns(Sueoka and Cheng, 1962) at room temperature. Nucleic acids were eluted under mild air pressure in a stepwise manner with from 0.3 to 1.2M NaCl in 50mM sodium phosphate buffer (pH 6.3). Twelve fractions of 3 ml each were collected for each saline concentration and ultraviolet absorption was measured at 255mμ in a Beckman DU spectrophotometer.

RESULTS

In order to obtain sequential rather than random cellular events with regard to nucleic acid synthesis during cyst germination, experiments were designed to obtain phased germination of a cyst culture. The results of an experiment utilizing the previously described method are presented in Fig. 1. The total number of cells as determined by viable cell counts was observed to double at 6 and 9 hours after addition of the carbon source. Direct cell counts made with a Petroff-Hauser counting chamber were in close correlation with the viable cell counts. Phase microscope observations were in close agreement with the earlier observations of Wyss, Neumann and Socolofsky (1961).

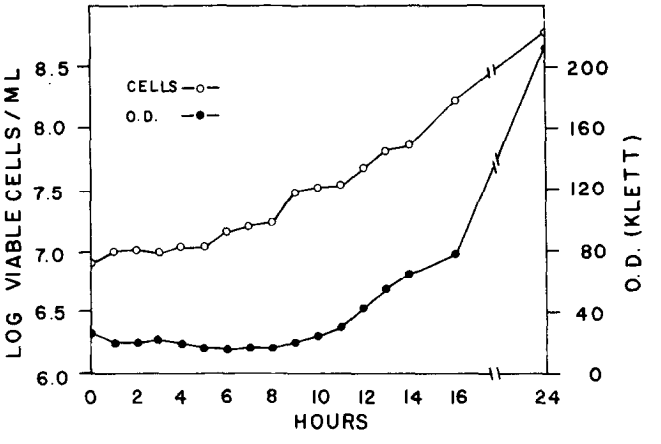

Fig. 1. Phased germination of Azotobacter cysts. Cyst cultures were phased by the temperature shift method: 1 C for 50 minutes then 33 C for 15 minutes, followed by the addition of sucrose. Viable and direct cell counts revealed a two-fold increase in cell numbers at 6 and 9 hours.

Extracts of dormant cysts had a nucleic acid elution pattern as illustrated in Fig. 2. Other bacterial systems exhibit similar elution patterns (Midgley and McCarthy, 1962). Peaks I, III and IV

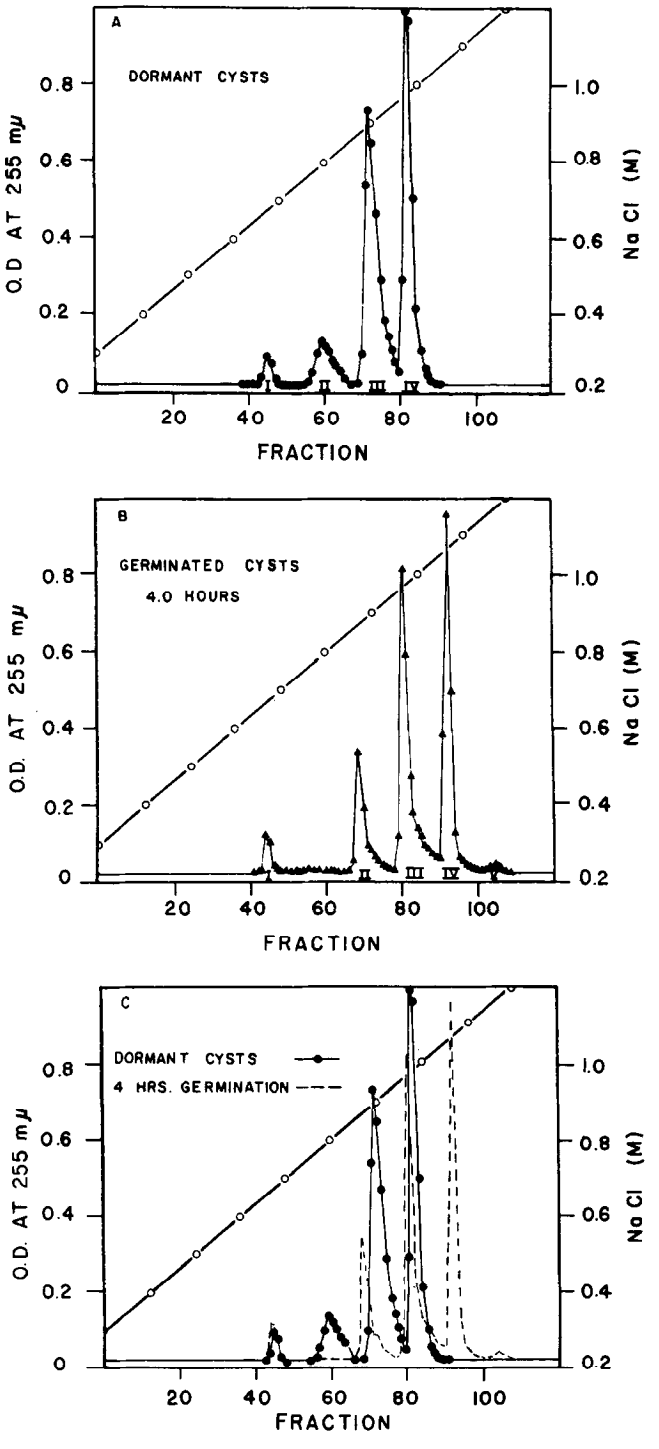

Fig. 2. Elution patterns (from MAK columns) of nucleic acids extracted from dormant cysts (A) and 4 hour germinated cysts (B) Nucleic acids were determined by absorbance at 255 mμ. In these patterns, peak I represents sRNA; peak II, DNA; peak III, 16S rRNA; peak IV, 23S rRNA; and peak V, unidentified RNA; O, sodium chloride gradient. Peaks II, III and IV exhibited a shift in elution pattern after 4 hours germination as seen in graph C which is a composite of graphs A and B.

contained RNA and peak II contained DNA as revealed by RNA determinations by the orcinol method (Schneider, 1957) and DNA determinations by either the Keck modification of the Ceriotti reaction (Keck, 1956) or the method of Burton (1968). It has been shown in other systems that peak I contains transfer RNA and peaks III and IV contain 16S and 23S RNA, respectively (Sueoka and Cheng, 1962). Similar elution patterns were obtained for samples extracted at 2 hour intervals after the start of germination. Nucleic acids from each interval were extracted and chromatographed at least three separate times and the results obtained were found to be closely reproducible. When nucleic acids from a 4 hour culture were chromatographed, it was noticed that peaks II, III and IV eluted at higher saline concentrations than did the analogous peaks at 0 and 2 hours. This finding is illustrated in Fig. 2. Peak I elutes at 0.6M saline while peaks II, III and IV elute at approximately 0.1M higher saline concentration than the analogous peak from dormant cysts. Nucleic

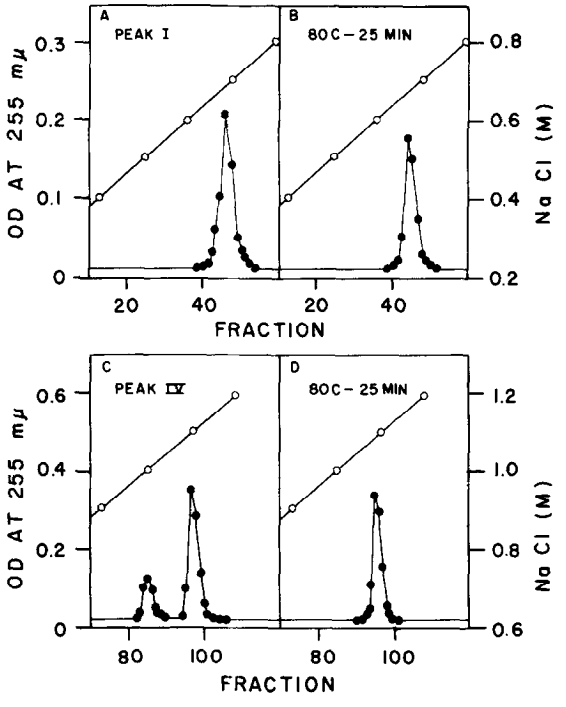

Fig. 3. Co-chromatography (from MAK columns) of pooled fractions from analogous peaks of dormant cyst and 7.5 hour germinated cyst nucleic acid extracts. Peak I eluted at 0.6M NaCl before and after heating (A,B). Peak IV eluted as two distinct peaks prior to heating (C), but only one peak was detected after heating the pooled fractions (D).

acid extracts from 4, 6 and 7.5 hour germinated cyst cultures and log-phase vegetative cells exhibited similar elution profiles. When variations in the extraction procedure were employed, there were no significant differences in the various elution profiles. As seen in Fig. 2, a small fifth peak, which eluted after peak IV, was first detected at 4 hours and also appeared at 6 and 7.5 hours and in vegetative cell preparations. Peak V was found to contain RNA but there is no information concerning the nature and character of this peak.

In conjunction with a working hypothesis that changes in elution profiles were the result of differences in configuration, the following experiments were performed. The fractions containing the analogous peaks from dormant cysts and 7.5 hour germinated cyst preparations were pooled and again chromatographed. The results of a typical experiment are presented in Fig. 3. Detection of two distinct peaks supported the contention that configurational differences resulted in these elution patterns. Similar pooled fractions were then heated at 80 C for 25 minutes and chromatographed. As seen in Fig. 3, a single peak was detected. Identical results were obtained for peaks II and III, although peak I from cysts and germinated cysts did not behave in this manner, as illustrated in Fig. 3.

DISCUSSION

The Azotobacter cyst is a unique resting cell which can survive in an environment unsuitable for normal growth. The studies of Parker and Socolofsky (1966) indicate that the cyst coat structure is primarily responsible for the survival and resistance properties of the cyst. The evidence presented in this communication suggests that the nucleic acids of the cyst may also adapt to the conditions of a deleterious environment.

As observed by Wyss, Neumann and Socolofsky (1961), the nuclear material of the cell undergoes compaction during cyst formation. The size of the central body decreases from compaction during this formative period, yet it increases in size during the 6 to 8 hour germination process. Changes in MAK column elution profiles have been detected from nucleic acids extracted from dormant cysts and cysts at various times during germination. After 4 hours, the DNA and ribosomal RNA are seen to elute at a one step higher saline concentration than the analogous peaks from dormant cysts and 2 hour germinated cysts. The 4S RNA peak does not change in elution pattern. When the analogous peaks were pooled and re-chromatographed, two distinct peaks were detected. After heating the pooled fractions,

only one peak was apparent. Heating the nucleic acids for a short time under mild acid conditions has been observed by Bock (1967) to rupture a small number of the hydrogen bonds. It is suggested from our findings that rupture of hydrogen bonds unique to the cyst DNA and ribosomal RNA occurs, which results in a configuration similar to those of vegetative cells and late germinated cysts.

These results suggest that during the encystment process and subsequent compaction of the central body and nuclear material, the configuration of the DNA and ribosomal RNA is modified. Such a change in configuration could contribute a survival advantage by protecting the nucleic acids from possible denaturation in a deleterious environment. The transfer RNA did not exhibit this effect suggesting that these somewhat smaller molecules are not modified during the encystment process. Change in elution profiles in other bacterial systems have also been attributed to changes in configuration, quite possibly in the secondary and/or tertiary structure of the polynucleotide chains (Kaji and Tanaka, 1967; Stern, Zutra and Littauer, 1969). Stafford and Donnellan (1968) have reported photochemical evidence for differences in the configuration of spore and vegetative cell DNA.

The detection, after 4 hours germination, of a small peak eluting after the 23S RNA peak is of interest. This peak is an RNA peak and may represent messenger RNA. Doi and Igarashi (1964) studied spore messenger RNA fractions and these were found to elute after the 23S RNA. Failure to detect this peak prior to 4 hours germination may indicate low messenger RNA content in the dormant cyst and during early germination.

The results of our investigation indicate that in addition to the survival advantage conferred by the protective cyst coat structure, other advantageous events may occur during cyst formation. Changes in nucleic acid configuration in conjunction with central body compaction may also represent an additional survival mechanism. Such changes in the intracellular environment may enhance the ability of the cyst to survive in a deleterious external environment. Quantitative changes in the nucleic acids during the germination process will be reported in a future communication.

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