Patterns of Cell Polarity and Chromosome Segregation in Chains of Sporulating *Bacillus megaterium*

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The asymmetry of the DNA duplex due to polynucleotide strand complementarity could be the molecular basis of cell polarity in spore-forming bacteria. To test this possibility, the relationship of DNA strand segregation to the spore location pattern in chains of sporangia was investigated in *Bacillus megaterium*. Spores containing one chromosome labelled in one of the complementary strands were formed from cells that had been allowed to segregate pulse-labelled chromosomes in minimal medium at 30 °C. A second crop of spores was then formed from cells originating from the labelled spore population. The second generation spores inherited labelled strands from the first spore population by random segregation. In contrast, the patterns of spore positions in sporangial chains were non-random. Furthermore, the non-randomness of patterning was stable and was unaffected by growth temperature (15 to 37 °C) or by enrichment of the minimal medium used in the segregation experiments. Since the pattern of DNA strand segregation is random and the spore location pattern in chains of sporangia is non-random, the asymmetry of the DNA duplex cannot be the determinant of cell polarity.

INTRODUCTION

While cell polarity and the development of pattern are usually regarded as unique characteristics of eukaryote development, they are also exhibited by prokaryotes which possess primitive developmental systems (Konigsberg, 1979). The spore-forming bacilli produce morphologically asymmetric cells when they sporulate. A single intracellular spore is initiated at one pole of the rod-shaped sporangium by means of an asymmetric cell division. In *Bacillus megaterium* the spore loci in chains of sporangia are non-randomly located and form a geometrically simple pattern (Hitchins, 1975, 1978*b*). The occurrence of cell polarity in a primitive organism such as *B. megaterium* makes it a useful prototype for investigating the principles that may be fundamental to these developmental phenomena in higher organisms.

The spores in chains of *B. megaterium* sporangia are located distally to the most recent vegetative cell division septa (Hitchins, 1975; Johnstone & Ellar, 1978). This suggests that there is a polarity in the presporangium that influences the subsequent position of the asymmetric prespore septum. The source of the asymmetry and the cell polarity observed in chains of bacteria could be related to the mechanism of cell division since the asymmetric septation of sporulation may be a modification of the symmetric septation that occurs during vegetative cell division (Doi, 1977; Ellar, 1978; Freese *et al.*, 1978; Hitchins, 1978*a*; Hitchins & Slepecky, 1969). The patterns of spore loci demonstrate that there is a regular

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topological relationship between the asymmetric sporulation septa and the symmetric vegetative septa in chains of spore-forming cells. Therefore, it is logical to search initially for a source of asymmetry in the cell division process that could be expressed when sporulation is induced by nutrient deprivation. One such source is the DNA duplex which is structurally asymmetric. This asymmetry could lead to differential behaviour and expression of the sporangial chromosomes prior to asymmetric septation. DNA asymmetry would thus be the basis for the subsequent differences between the two ends of the sporangium. One way in which the asymmetry could be manifested is as a non-random pattern of DNA segregation with respect to chromosome age.

A previous study showed that old chromosomes of *B. megaterium* segregate into spores and mother cell compartments of sporangia at random and independent of the spore loci patterning in sporangial chains (Hitchins, 1978*b*). However, age-independent chromosome segregation could still be compatible with an age-dependent strand-specific segregation pattern (Hitchins, 1976). Thus, the chemical specificity of the complementary polynucleotide strand containing the marker label in an old chromosome may be an important factor in the detection of non-random segregation. The results of experiments to test this hypothesis are reported here. They show that segregation into spores is random with respect to strand-specificity, suggesting that the asymmetry of DNA due to strand complementarity cannot account for patterns of spore loci in chains of *B. megaterium*.

METHODS

Bacteria. The organism used was a thymine auxotroph of *Bacillus megaterium* ATCC 19213.

Growth and sporulation conditions. Cultures were prepared by inoculating heated (70 °C; 30 min) spores into 50 ml sucrose/salts medium containing 0.5 % (w/v) sucrose (Slepecky & Foster, 1959), 100 µg L-alanine ml⁻¹, 100 µg inosine ml⁻¹ and 50 µg thymine ml⁻¹. Actively growing cells were sedimented and resuspended in 25 ml sucrose/acetate/salts sporulation medium containing 0.1 % (w/v) sucrose, 0.1 % (w/v) sodium acetate and 2 µg thymine ml⁻¹. Cultures were shaken in side-arm flasks in a water bath at 30 °C. Culture turbidity was monitored spectrophotometrically at 600 nm.

Spore frequency and polarity indices. The sporulation frequency was expressed as the percentage of cells containing phase bright spores at peak refractivity; at least 100 cells per sample were examined.

Polarity was measured as described previously by an index defined in percentage units (Hitchins, 1978*b*). The polarity index is the percentage of spores occupying the positions expected when each spore is preferentially located at the old end of its sporangium as in *B. megaterium* (Hitchins, 1975). Arbitrarily beginning at the left end of a chain, the successive spore loci will alternate regularly among the left and right sporangial poles as a chain is inspected from left to right. Thus in Fig. 1 (top row) the left, right, left, right pattern of terminal spore location corresponds to a polarity index of 100 %, i.e. an exact correspondence with the ideal pattern. Asporogenous cells and chains with odd numbers of sporangia can lead to an underestimate of the index, but this error is insignificant for *B. megaterium* grown in sucrose/salts medium (Hitchins, 1975, 1978*b*). Patterns differing from the *B. megaterium* pattern will yield polarity indices much less than 100 %, as shown in the other examples in Fig. 1.

Collection and processing of sporangia and spores. Samples of young sporangia were collected about 1.5 h after growth ceased. The samples were hydrolysed with warm alkali or hot acid as described below. Spore samples were obtained after sporangial autolysis, unless stated otherwise. The spores were incubated at 35 °C for 60 min with 200 µg chicken egg-white lysozyme ml⁻¹ (EC 3.2.1.17) and 100 µg bovine pancreatic deoxyribonuclease ml⁻¹ (EC 3.1.21.1) to release and degrade any residual extrasporal DNA present in either the unlysed cells or the culture fluid. The enzyme-treated spores were then subjected to the same chemical hydrolyses as the sporangia. Identical amounts of the hydrolases were added to the sporangia just after addition of the acid or alkali but prior to the incubations in order to maintain a constant concentration of added protein in all samples.

Pulse radiolabelling of DNA. Cells in the exponential phase of growth were labelled with [*methyl*-³H]thymidine. The cells in a 50 ml culture with a density corresponding to 0.2 *A*₆₀₀ units were concentrated by centrifugation at 5 °C. The cell pellet was suspended in 2 ml sucrose/salts medium containing [*methyl*-³H]-thymidine [0.1 mCi; 52 Ci mmol⁻¹ (1.92 TBq mmol⁻¹)] and excess sucrose (0.5 %, w/v) and the suspension was incubated at 30 °C for 8 min. Labelled cells were used immediately for segregation experiments.

Assay of radiolabelled DNA. The amount of label in the DNA of sporangia and spores was expressed as

Pattern	Polarity (%)
100
50
67
33
71
0

Fig. 1. Pattern types and the corresponding polarity indices. Polarity indices were calculated as described in Methods. The indices are a measure of the direction of sporangial polarization and hence patterning. An example of 100% polarity is shown in the top panel and is the standard reference pattern since it occurs with high frequency in *B. megaterium*. Although the chains with three sporangia have low indices (33 or 67) the patterns are consistent with the standard pattern, as explained elsewhere (Hitchins, 1975). Such chains were infrequent enough not to lower the average polarity index below 85% in the growth conditions used in the present study. The other examples are illustrative only.

the radioactivity (c.p.m.) in the alkali-resistant portion of the hot acid-soluble macromolecular fraction. The amount of label in the DNA equals the radioactivity in the cold acid-precipitable part of the alkali-resistant fraction minus the radioactivity in the cold acid-precipitable part of the hot acid-resistant fraction.

The amount of cold acid-precipitable material in the alkali-resistant fraction was determined as follows. Quadruplicate samples of suspensions of the various cell forms were mixed with equal volumes of 2 M-KOH and incubated at 35 °C for 20 h. The alkali digests were cooled to 0 °C and neutralized with cold 2 M-HCl. Each neutralized digest was mixed with an equal volume of cold 10% (w/v) trichloroacetic acid (TCA), and after 30 min at 0 °C the precipitate was collected on a nitrocellulose filter (Millipore, 0.45 µm pore size). The loaded filters were washed three times with 5 ml cold 5% TCA containing 100 µg thymine ml⁻¹ and 100 µg thymidine ml⁻¹.

The amount of cold acid-precipitable material in the hot acid-resistant fraction was obtained by heating quadruplicate samples with 0.5 M-HClO₄ at 80 °C for 3 h. Hydrolysed samples were cooled to 0 °C and mixed with equal volumes of cold 10% TCA. After 30 min at 0 °C the precipitates were collected and processed as described above. Dried filters were radioassayed in glass vials containing Econofluor scintillation fluid (New England Nuclear).

Segregation procedure. Cells containing pulse-labelled DNA were grown in sucrose/acetate/salts medium for 1 to 6 generations before being permitted to sporulate. The final density of the sporangial suspensions was similar in order to maintain an approximately constant dry weight on each filter. This was achieved by varying the initial cell densities while maintaining a constant initial concentration of growth limiting substrate (sucrose). Batches of sporangia were produced that contained pulse-labelled chromosomes of different ages. Chromosome ages were defined as the number of turbidimetrically determined cell generations of post-labelling segregation.

The DNA made subsequently to DNA pulse-labelled with [*methyl*-³H]thymidine was differentially and continuously labelled with [2-¹⁴C]thymine [1 µCi; 53 mCi mmol⁻¹ (1.96 GBq mmol⁻¹)]. By using a double label technique no correction for sporulation frequency was required to estimate the relative amount of pulse-labelled [³H]DNA segregating into spores. The ratios of ³H/¹⁴C in spore DNA and in the total DNA of the young sporangia were compared. Any preferential segregation of pulse-labelled [³H]DNA into the mother cell or the spore would be indicated by a change in the ³H/¹⁴C ratio. Segregation of the [¹⁴C]DNA to the level of one strand per cell cannot occur because exogenous [¹⁴C]thymine is present continuously during the segregation of the [³H]DNA. Thus the ³H/¹⁴C ratio is proportional to the specific radioactivity of [³H]DNA per unit mass of total DNA.

Fig. 2. Demonstration of chromosome age independence of segregation of pulse-labelled chromosomes into spores. Cells containing chromosomes pulse-labelled with [*methyl*- ^3H]thymidine were permitted to segregate DNA strands for up to five generations in sucrose/acetate/salts medium at 30°C . During segregation the cells were continuously labelled with [$2\text{-}^{14}\text{C}$]thymine. The ratio of the $^3\text{H}/^{14}\text{C}$ value in spore DNA to the $^3\text{H}/^{14}\text{C}$ value in the DNA of young sporangia after different periods of segregation was calculated (a). A value of unity indicates no difference between the $^3\text{H}/^{14}\text{C}$ ratios of the DNA of spores and of young sporangia. The proportion of sporangial DNA-label segregation into spores (b) was calculated from the ^3H radioactivity only. The frequency of sporulation was high but was not measured accurately. The results from two experiments are distinguished by the different symbols. The curves are least squares regression lines.

RESULTS

Two changes in the chromosome labelling protocol facilitated the test for strand-specific segregation into spores. First, cells had previously been pulse-labelled with radioactive thymine for 0.4 generations (Hitchins, 1978*b*). In this study labelling with radioactive thymidine increased the level of incorporation of radioactivity during a reduced pulse time of 0.11 generations. The old and new labelling protocols were found to yield similar results. Thus, as the ages of the labelled chromosomes increased to 5.5 generations their probability ($P = 0.4$ to 0.5) of segregating into spores did not change significantly. Second, a double radiolabel segregation technique was employed so that the frequency of sporulation did not have to be determined. Chromosomes were pulse-labelled with [*methyl*- ^3H]thymidine and the DNA formed during the subsequent segregation period was continuously and differentially labelled with [$2\text{-}^{14}\text{C}$]thymine. As shown in Fig. 2(a), there was only a slight decrease in the probability for pulse-labelled (^3H) chromosomes to segregate into spores relative to the younger continuously labelled (^{14}C) chromosomes. For comparison of the single and double label methods, Fig. 2(b) demonstrates the same result using only the pulse-label data from the double label experiment and correcting it for the frequency of sporulation.

Strand-specificity of chromosome segregation

Since the results of experiments using the new and old labelling protocols were similar, the double label technique was used to test for a strand-specific segregation pattern. Exponentially growing cells were pulse-labelled with [*methyl*- ^3H]thymidine for 0.11

Table 1. Segregation of pulse-labelled DNA during a second cycle of sporulation

Young sporangia and spores were obtained and processed as described in Methods. The radioactivity (c.p.m.) in the mother cell fraction was obtained from the difference between that in mature sporangia containing refractile spores and that in the spores released from those sporangia by lysis with lysozyme. The ratio $^3\text{H}/^{14}\text{C}$ represents the ^3H radioactivity in the old pulse-labelled DNA divided by the ^{14}C radioactivity in newer continuously labelled DNA. In the fourth column, the calculated ratios in the various cell forms are for a random strand segregation pattern; the calculations are based on the averages of the relative empirical ratios for young sporangia in the two experiments taking the values for sporangia as unity. In the fifth column, the theoretical average ratios are presented for a non-random segregation model in which chromosomes with old strands segregate exclusively into the second generation spores.

Cell form	Empirical* and theoretical average $^3\text{H}/^{14}\text{C}$ ratios			
	Expt 1	Expt 2	Relative average	Theoretical average
Young sporangium	0.447 $\pm$ 0.090†	0.379 $\pm$ 0.067	1.00	1.00
Spore	0.382 $\pm$ 0.135	0.340 $\pm$ 0.059	0.88	2.00
Mother cell	—	0.393 $\pm$ 0.096	1.04	0.00

* Results are the averages of those from four sets of samples; the ranges are the 95 % confidence limits.

† Average and 95 % confidence limits for three sets of samples. Inclusion of the fourth set would give a value of 0.378 ± 0.291 . Thus, the fourth set was omitted because it resulted in an atypically high confidence limit: the average confidence limit was ± 0.09 .

generations. The labelled strands were segregated during further growth in unlabelled medium. Cells were then permitted to sporulate. The spores were released, cleaned and then induced to germinate and form a growing culture. During growth, the new DNA formed was labelled with [2- ^{14}C]thymine. After a second cycle of sporulation the second crop of spores was isolated and its $^3\text{H}/^{14}\text{C}$ ratio was compared with that of the corresponding sporangia. As shown in Table 1, there was no tendency for old [^3H]DNA to be selectively incorporated into spores since the proportion of old DNA to new DNA in spores was the same as in their presporangia. This implies that DNA segregation during sporulation is not strand-specific. If the preseggregated labelled strands in the first spore crop were composed of only one type of complementary strand, it would be expected that they would have also preferentially segregated into the second spore crop with a concomitant enrichment of old DNA relative to new DNA.

Constancy of cell polarity

The marked regularity of the spore location pattern in *B. megaterium* is well established (Hitchins, 1975; Johnstone & Ellar, 1978). The pattern is invariant in the minimal and enriched growth media that have been examined so far, in contrast to the variability seen in *B. subtilis* (Dunn & Mandelstam, 1977). In the present study the polarity index was 85 % at 30 °C in sucrose/acetate/salts medium and it was not significantly affected by enrichment with casein hydrolysate and yeast extract. These values are underestimates since the sporulation frequency, though typically high (90 %), was less than 100 %. Furthermore, the polarity index was essentially unaffected by growth temperature in the sporulation temperature range 15 to 37 °C. The maximum growth temperature was about 46 °C, but above 37 °C the sporulation frequency declined sharply, making it difficult to estimate polarity indices. In contrast, the minimum temperature at which growth was detectable was 15 °C and sporulation still occurred with high frequency, although spore formation took longer.

DISCUSSION

It was demonstrated previously that there is no preferential segregation of young versus old chromosomes into the spore and mother cell compartments of the asymmetrically

divided sporangium of *B. megaterium* (Hitchins, 1978*b*). The results of the present study show that when the nature of the pulse-labelled strand in an old chromosome is considered, segregation is still random. Although chromosome segregation is random, the spores into which the chromosomes segregate are non-randomly distributed in sporangial chains. Therefore, the patterns of chromosome segregation and spore location are not coincident. The spore location patterning is based on a polarization of the presporangium. The direction of the polarization in *B. megaterium* involves spore localization at the pole distal to the one formed by the symmetric division that occurred just prior to sporulation. The results show that non-random polarization of the sporangium cannot be correlated with one asymmetric facet of DNA, namely, the asymmetry due to strand complementarity.

The results of this study do not rule out DNA strand asymmetry being a contributing factor in cell polarity determination. However, a multi-factor process for polarity determination would be complex and at present it is more economical to look for another determinant of polarity at the non-DNA level. The sporangial cytosol is believed to be homogeneous until some time after the asymmetric division (Freese & Freese, 1977). Thus the cytosol is not likely to be polarized. This leaves the cell envelope as the most likely source of polarity, and hence patterning, as already suggested by Dunn & Mandelstam (1977). If so, the sporangial wall and/or membrane would have to retain the information necessary to determine that the spore is located at the end of the sporangium which is old with respect to the end formed by the last cell division septum. In view of the current lack of knowledge of the extent and distribution of topological heterogeneities in the wall and membrane, finding out the way in which the envelope layers could determine polarity presents a formidable problem.

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