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Membrane Enrichment of Genetic Markers Close to the Origin and Terminus During the Deoxyribonucleic Acid Replication Cycle in *Bacillus subtilis*

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A temperature-sensitive *Bacillus subtilis* initiation mutant was used to achieve one cycle of synchronized deoxyribonucleic acid (DNA) replication. Markers near the origin of replication and the terminus were assayed for association with the cell membrane at intervals during the DNA replication cycle. DNA near the origin and terminus was found to be enriched in the membrane fraction throughout the DNA replication cycle. The magnitude of membrane enrichment of origin and terminus markers varied coincidentally, possibly as a consequence of incubating the cells at 45°C.

Cell membrane enrichment for DNA containing genetic markers near the replication origin and terminus has been observed in exponentially growing cells of *Bacillus subtilis* by Sueoka and Quinn (16). Pulse-labeling at the origin and subsequent chase showed that the label was associated with the membrane and remained so during DNA replication (16; Winston and Sueoka, unpublished data). These results were interpreted to mean the origin and probably the terminus are permanently attached to the membrane. Whereas there have been a number of papers confirming the membrane association of the origin and terminus in *B. subtilis* (10, 14, 20, 21) and in *Escherichia coli* (4, 11), the permanency of the association throughout the cell cycle has been questioned experimentally and theoretically (3, 7, 18, 19). The temporary membrane association of the origin and the terminus has been implied to play a part of the regulatory process of replication initiation and replication termination. On the other hand, there are results which support the membrane-chromosome association throughout the chromosome replication cycle (6, 12, 13).

In this study, we examined the membrane association of the origin and the terminus at various times within one cycle of DNA replication by using a temperature-sensitive *B. subtilis* initiation mutant to obtain synchronous DNA replication. Special effort in designing experiments was made to prevent the occurrence of dichotomous replication (premature initiation). Evidence is provided that DNA around the origin and the terminus remains associated with the membrane throughout the DNA replication cycle. The extent of membrane enrichment of markers near the origin and near the terminus varies in a similar fashion within the replication cycle.

MATERIALS AND METHODS

Strains. Cell synchrony was achieved by utilizing the temperature-sensitive initiation mutant *B. subtilis* 168 *trp thy dna-1*(Ts) (17); 34°C was used as the permissive temperature for initiation and 45°C was the nonpermissive temperature (17). *B. subtilis* 168 *leu-8 metB5 purA16* and *B. subtilis* 168 *leu-8 thr-5* (22) were used as recipients for transformation.

Media. Synchronization medium consisted of medium C of Spizizen (1) supplemented with 500 μg of Casamino Acids, 50 μg of L-tryptophan, 2 μg of thymine, and 10 μCi of [^{3}H]thymine (Amersham-Searle; 20 Ci/mmol) per ml. Penassay medium is antibiotic medium no. 3 (Difco). Bott and Wilson's transformation medium (2) was supplemented with the auxotrophic requirements of the recipient strain (50 μg/ml) plus 50 μg of histidine and tryptophan per ml.

Growth and synchronization of cells. The growth and synchronization scheme is shown in Fig. 1. Strain 168 *trp thy dna-1*(Ts) was grown overnight at 34°C in Penassay medium supplemented with 50 μg of L-tryptophan and 10 μg of thymine per ml. The cells were harvested by centrifugation and resuspended in synchronization medium at 1.7×10^7 cells per ml. They were grown at 34°C to 7×10^7 cells per ml. The generation time varied between 60 and 70 min. An aliquot of these exponentially growing cells was used to inoculate 70 ml of prewarmed synchronization medium (45°C) at 1.4×10^7 cells per ml. The culture was grown at 45°C for 1 h to allow chromosome elongation to proceed to completion. The cells were then sampled (zero time point) and harvested by filtration.

The filtered cells were rapidly washed with synchronization medium (34°C) and immediately resus-

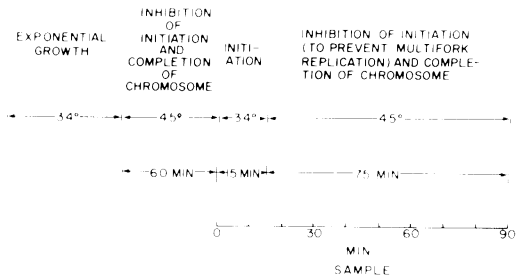

FIG. 1. *Growth and synchronization protocol for 168 trp thy dna-1(Ts). The cells were grown and incubated in medium C (1) supplemented with Casamino Acids, L-tryptophan, 2 μg of thymine per ml, and 10 μCi of [^{3}H]thymine (Amersham-Searle, 20 Ci/mmol) per ml. After two generations of exponential growth at 34°C (generation time, 60 to 70 min), the culture was diluted from 7 × 10^7 to 1.4 × 10^7 cells per ml. The cells were incubated at 45°C for 1 h, harvested by filtration, and resuspended in fresh medium. The culture was shifted to 34°C for 15 min to initiate a new round of chromosome replication and then incubated at 45°C for 75 min to permit elongation without the possibility of dichotomy. Samples were taken for the analysis of the extent of chromosome replication and the enrichment of markers in membrane-bound DNA at 10-min intervals, starting at the time of the shift from 45 to 34°C.*

pended at the same concentration in prewarmed synchronization medium. The resuspended cells were incubated at 34°C for 15 min and then incubated for an additional 75 min at 45°C. The cells were sampled every 10 min from zero time. Sampling consisted of the following steps. (i) The turbidity of the culture was recorded. (ii) A 0.1-ml aliquot was precipitated with 10% cold trichloroacetic acid, and cells were collected on a GF/C filter and counted in a liquid scintillation counter to determine incorporation of [^{3}H]thymine into DNA. (iii) Ten microliters of 0.1 M KCN was added to a 1-ml aliquot of cell suspension. The cells were harvested by centrifugation, quick-frozen in dry ice-ethanol, and stored at −80°C. This aliquot was used as a source of DNA to check synchrony. (iv) Fifty microliters of 0.1 M KCN was added to a 5-ml aliquot of cell suspension, the cells were harvested by centrifugation, and the cell samples were frozen to provide material for the preparation of DNA membrane complex.

Preparation of DNA for testing synchrony. The frozen cell pellets were thawed at room temperature and resuspended in 1 ml of 0.15 M NaCl–0.1 M EDTA, pH 8.0. Lysozyme was added to 0.5 mg/ml, and the suspension was incubated at 37°C for 20 min. Sodium dodecyl sulfate was added to a final concentration of 0.5%, and the 37°C incubation was continued for 15 min. Predigested pronase (37°C, 2 h in 0.15 M NaCl–0.015 M EDTA, pH 8.0) was added to the lysate to a final concentration of 0.5 mg/ml, and the lysate was incubated at 50°C for 1 h. The lysate was gently shaken with an equal volume of phenol saturated with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The phases were separated by centrifugation, and the aqueous phase was extracted two times with twice its volume of anhydrous diethyl ether. The residue of ether was removed from the aqueous phase by bubbling nitrogen through the sample and then evacuating the sample in a desiccator for 15 min. The sample was then dialyzed overnight at 4°C against two 1-liter changes of sterile 1× SSC.

Lysis of cells and preparation of transforming DNA from the DNA-membrane fraction. The procedure for cell lysis has been described (5). The volumes given are for a 5-ml pellet from a culture whose turbidity was 35 Klett units. The volumes of lysis reagents were adjusted by the ratio of the sample turbidity to 35 Klett units in an attempt to insure uniformity of lysis conditions. The frozen pellet was thawed at room temperature and resuspended in 0.2 ml of 0.05 M Tris–0.1 M EDTA–0.5 M sucrose, pH 8.0, plus 0.075 ml of lysozyme solution (3 mg/ml) in 0.02 M Tris, pH 8.0, pH 8.0, and 0.020 ml of 0.1 M KCN. The suspension was incubated at 0°C for 1 h. The protoplasts were then burst by the rapid addition of 0.75 ml of 0.15 M sucrose–0.05 M Tris–0.1 M EDTA, pH 8.0. The lysate was incubated for 30 min at 32°C. The clear, viscous lysate was sheared in a 13-mm screw-cap tube by mixing at the highest speed of a Vortex mixer for 1 min. The sheared lysate was applied to a 4-ml CsCl sucrose double gradient and treated as previously described (15). Twenty fractions were collected from each gradient, and the DNA distribution in the gradient was determined by precipitating 20-μl aliquots of each fraction with cold 10% trichloroacetic acid, filtering, and counting the precipitates in a liquid scintillation counter. Tubes containing membrane fractions (both M_1 and M_2; 15) and free fraction were pooled, and DNA was partially purified from the pooled M and F fractions of each time point. Pooled fractions were brought to a total volume of 2 ml with 0.15 M NaCl–0.1 M EDTA, pH 8.0, and dialyzed against the same buffer overnight at 4°C. DNA from each fraction was partially purified exactly as described for whole-cell lysates.

Transformation. Transformations were performed as described by Bott and Wilson (2). Selection plates contained 50 μg of nitrogenous base requirements and 100 μg of L-amino acids per ml.

RESULTS

Synchronization. The incorporation of [^{3}H]thymine into DNA in synchronized 168 *trp thy dna-1*(Ts) is shown in Fig. 2. The approximate doubling of radioactivity is consistent with the occurrence of one round of DNA replication. Synchrony was examined by using whole-cell lysate DNA from the ten time points after the first 45°C incubation to transform *leu-8-metB5-purA16*. This strain carries markers near the origin of the chromosome (*purA16*), near the middle (*leu-8*), and close to the terminus (*metB5*) (9). The marker ratios at the time of the shift from 45 to 34°C should approximate those of spore DNA in which all markers are equally frequent (8) if the *dna-1* chromosomes are completed during the first 45°C incubation.

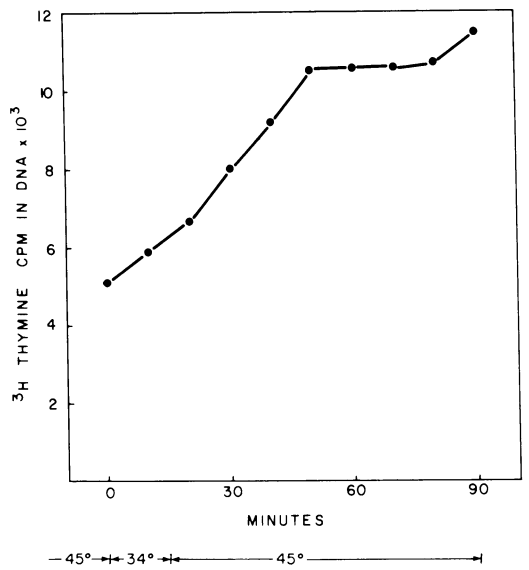

FIG. 2. *Incorporation of [^{3}H]thymine into DNA in synchronized 168 trp thy dna-1(Ts). The cells were uniformly labeled with [^{3}H]thymine during exponential growth and subsequent incubations. Aliquots of 0.1 ml were taken at 10-min intervals starting with the shift from 45 to 34°C and precipitated with 10% trichloroacetic acid. The precipitate was collected on GF/C filters and counted in a liquid scintillation counter.*

The *purA16/metB5* marker ratio normalized to that of spore DNA at this time was 1.2, indicating that most of the chromosomes had completed the *metB5* marker. This result is consistent with our previous data (17). Failure of the marker ratio to drop to 1 may result from a difference in elongation rates among cells or from elongation having stopped prematurely in a small fraction of cells. Calculations based on density transfer data (17) indicate that, in our experiments, no more than 10% of the cells did not complete replication at the time of the shift from 45 to 34°C. The marker ratio of the zero time point was used to normalize marker ratios from subsequent time points (Fig. 3). The normalized marker ratios for *purA16/leu-8* and *purA16/metB5* attained a value of approximately 2 at 20 min, indicating that *purA16* was replicating in a synchronous fashion and also showing that dichotomous replication did not occur. The normalized *purA16/leu-8* and *purA16/metB5* marker ratios fell to values slightly greater than 1 at later times, showing that most cells replicated *leu-8* and *metB5* by 90 min.

Changes in the membrane enrichment index of markers near the replication origin and terminus. Transformations were performed using membrane and free fraction DNAs from each time point. Membrane enrichment index were calculated as previously described (16). The membrane enrichment index of a marker, X is $(X_m/X_f)/(S_m/S_f)$, where X_m and X_f are the number of transformants of marker X from membrane-bound and free DNA, and S_m and S_f are the numbers of transformants of a standard marker. The standard marker was the middle marker *leu-8* in the experiments described in Fig. 4 and Table 1.

The marker *purA16* (near the origin) shows membrane enrichment throughout the DNA replication cycle. The magnitude of the membrane enrichment index varies but does not fall below 1.3 in experiment A and 1.4 in experiment B (Fig. 4). DNA for *metB5* (a marker near the

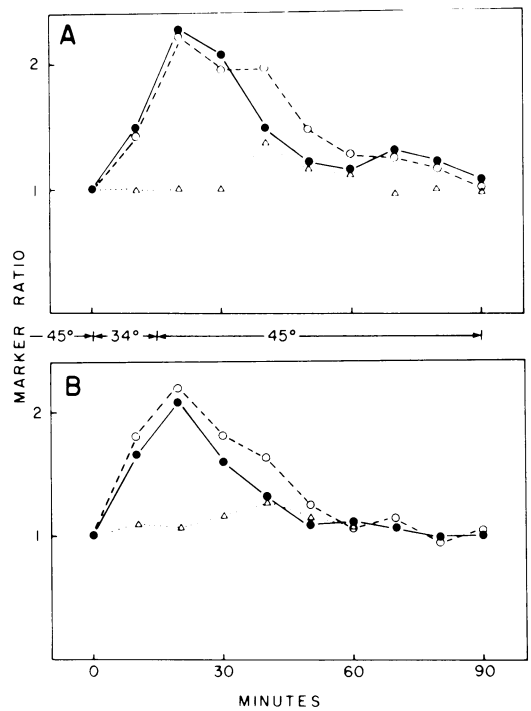

FIG. 3. *Chromosome replication in synchronized 168 trp thy dna-1(Ts) analyzed by marker ratios. DNA was partially purified from cell samples taken during the synchronized replication cycle and used to transform leu-8-metB5-purA16. Transformations were performed at least three times with each DNA sample, and the mean marker ratios are presented. Marker ratios were determined and divided by the marker ratio of the zero time point. Symbols: (○) ratio of purA$^+$ to metB5$^+$ transformants; (●) ratio of purA$^+$ to leu-8$^+$ transformants; (△) ratio of leu-8$^+$ to metB5$^+$ transformants. (A) and (B) represent results of two experiments.*

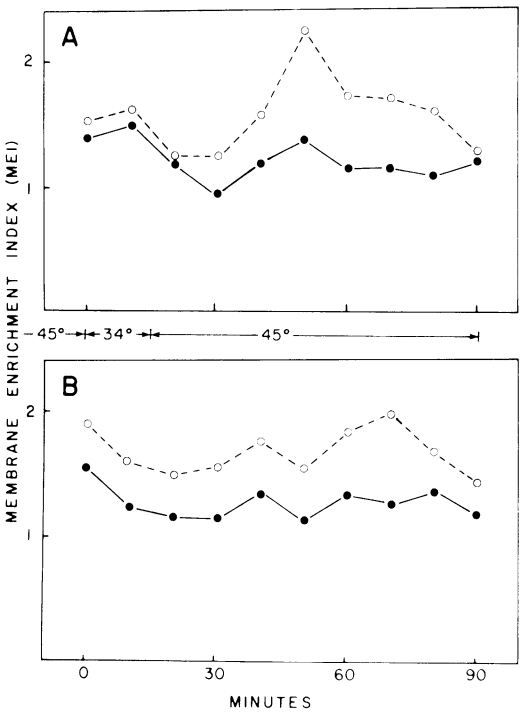

FIG. 4. *Membrane enrichment of purA16 and metB5 during one round of DNA replication in synchronized 168 trp thy dna-1(Ts). Membrane-bound DNA and free fraction DNA were separated on CsCl-sucrose double gradients, partially purified, and used to transform leu-8-metB5-purA16. Membrane enrichment index (MEI) were calculated as described in the text. Symbols:* (○) *MEI purA16;* (●) *MEI metB5. (A) and (B) represent results of two experiments.*

terminus) is also enriched in the membrane fraction throughout the replication cycle (Fig. 4) with the exception of the 30-min time point in experiment A, which we believe to be an artifact of preparation. Membrane enrichment of *purA16* and *metB5* appears to vary in a coincidental manner. The membrane enrichment index and standard errors of the mean for Fig. 4 are presented in Table 1.

The membrane enrichment of a marker should increase at the time of its replication and decrease at the time of replication of the standard marker, if DNA at the replication point remains bound to the membrane under our membrane isolation conditions. We calculated the membrane enrichment of *leu-8* using the earlier replicating middle marker *thr-5* as a standard (data not presented). No membrane enrichment of *leu-8* could be detected near its time of replication in the synchronized replication cycle. Since synchrony is quite good at the time of *purA16* replication, the membrane enrichment index of *purA16* should be at a maximum at the time of *purA16* replication if both replication origin DNA and replication point DNA remain bound to the membrane. The results show that the membrane enrichment index of *purA16* is not maximal when *purA16* replicates. Decreases in the membrane enrichment index of *purA16* and *metB5* were not observed at the time of *leu-8* replication. The data, therefore, do not provide evidence for the membrane binding of DNA in the vicinity of the replication fork under the present method of the complex isolation (high-salt condition).

DISCUSSION

Good synchrony of replication of early markers can be obtained by using *dna-1*. Synchrony appears to partially deteriorate before the replication of *leu-8*. A similar decay of synchrony has been observed for germinating spore DNA (8). The failure of the *leu/met* marker ratio to reach 2 could be explained by differences in the rates of DNA elongation among cells.

The reason for the variation of origin and terminus membrane enrichment index with time is not readily apparent. The variation cannot be explained by the progress of DNA replication since the membrane enrichments of *purA16* and *metB5* vary coincidentally. One possibility is that the DNA-binding properties of the membrane change as a consequence of shift in temperature. Temperature-dependent changes in the protein composition of the DNA-membrane fraction have been observed. For example, a 35,000-dalton polypeptide is missing from the DNA-membrane complex when *dna-1* is incubated at 45°C (5). The proportion of cellular DNA in the membrane fraction varied with the

TABLE 1. *Membrane enrichment indexes of purA16 and metB5 during one round of synchronous DNA replication*[a]

Time (min)	DNA prepn A (mean ± SE)		DNA prepn B (mean ± SE)	
	purA16	*metB5*	*purA16*	*metB5*
0	1.56 ± 0.16	1.43 ± 0.19	1.96 ± 0.10	1.60 ± 0.08
10	1.67 ± 0.28	1.53 ± 0.13	1.66 ± 0.18	1.26 ± 0.17
20	1.28 ± 0.05	1.21 ± 0.15	1.54 ± 0.05	1.19 ± 0.02
30	1.29 ± 0.02	0.96 ± 0.05	1.60 ± 0.06	1.17 ± 0.09
40	1.62 ± 0.12	1.22 ± 0.14	1.82 ± 0.19	1.38 ± 0.13
50	2.33 ± 0.23	1.44 ± 0.08	1.60 ± 0.06	1.17 ± 0.09
60	1.79 ± 0.20	1.18 ± 0.01	1.90 ± 0.11	1.38 ± 0.07
70	1.77 ± 0.26	1.18 ± 0.13	2.05 ± 0.05	1.30 ± 0.04
80	1.66 ± 0.32	1.13 ± 0.20	1.75 ± 0.08	1.40 ± 0.06
90	1.33 ± 0.19	1.25 ± 0.10	1.49 ± 0.08	1.21 ± 0.09

[a] The data are presented graphically in Fig. 4. Three transformations were performed using DNA from preparation A and five transformations were performed using DNA from preparation B. SE, Standard error of the mean.

temperature of incubation in these experiments (data not presented). Temperature shift may cause either the molecular composition or conformation of the membrane to change, thus altering the extent of recovery of membrane-bound DNA complexes during the isolation procedure, or, alternatively, the nature of the membrane-DNA complex may undergo changes within the cell cycle.

Membrane enrichment patterns of *purA16* and *metB5* (Fig. 4) do not support attachment-detachment models of the chromosome-membrane relationship proposed by Jones and Donachie (7), Worcell and Burgi (3, 19), and Winston and Matsushita (18). The first two groups based their conclusions upon results from the folded chromosome system of *E. coli*. They used amino acid starvation as a means to achieve a synchronized cycle of replication and examined the relative amounts of DNA in membrane-attached and free folded chromosomes. Their method of chromosome isolation prevented the detection of a more rapidly sedimenting folded chromosome attached to membrane, which was generated by amino acid starvation. This probably reflects the fact that amino acid-starved cells are more resistant to lysis than exponentially growing cells (6). In similar experiments, Ryder and Smith (12) and Korch et al. (6) detected the more rapidly sedimenting membrane-bound folded chromosome fraction. They concluded that DNA remained bound to the cell membrane throughout the replication cycle. This conclusion was supported by results from folded chromosome experiments where synchrony was achieved using a temperature-sensitive *dnaC* mutant (13).

Winston and Matsushita (18) investigated the permanency of *B. subtilis* origin and terminus attachment in experiments similar to our own except that synchrony was achieved by amino acid starvation or rifampin treatment. They proposed that their data could best be explained by a dynamic model of origin and terminus attachment and detachment (Matsushita and Winston, unpublished data) and concluded that protein synthesis is necessary for terminus release. A possible explanation for the differences between their data and ours is that the levels of membrane proteins necessary for the specific binding of origin and terminus DNA are altered in cells when protein synthesis is inhibited.

Failure to detect membrane enrichment of DNA near the replication fork may indicate that the binding of the replication point is weaker than the binding of origin and terminus DNA and that the binding was destroyed during sample preparation. Since the present preparation method involves high-salt conditions, ionic interaction of the replication point with the membrane is suspected as a major factor. Alternative possibilities are that replication point attachment is obscured by a temperature-dependent change in the stability of DNA-membrane binding, or that replication point attachment is too transient to be detected by the marker enrichment technique, or that synchrony was not adequate to permit detection of replication point binding.

ACKNOWLEDGMENTS

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