# REPLICATION OF *BACILLUS SUBTILIS* DNA DURING GERMINATION IN 5 BROMOURACIL CONTAINING MEDIUM AND MARKER MAPPING[1]

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IN the past few years investigations have been made on DNA replication during germination of *Bacillus subtilis* spores in 5-bromouracil (5-BU) containing medium (YOSHIKAWA 1965; OISHI and SUEOKA 1965; and WAKE 1967). Germination of spores in 5-BU containing medium has also been used for mapping of markers by the synchro-transfer method (OISHI and SUEOKA 1965 and O'SULLIVAN and SUEOKA 1967).

In this study we have chosen to examine the relationship between the replication of total DNA and the replication of individual markers during spore germination in 5-BU containing medium. An attempt was made also to map the *pur* (N) marker by the synchro-transfer method.

## MATERIALS AND METHODS

*Bacterial strains.* Spores of the thymine and indole requiring strain of *Bacillus subtilis* 168 *ind*$^-$, *thy*$^-$ described by FARMER and ROTHMAN (1965) were used in all germination experiments. The strains used as recipients in transformation experiments are listed in Table 1.

*Media and cultural procedures.* (1) Medium for spore preparations was TY agar (BRODETSKY and ROMIG 1965) supplemented with $10^{-5}$ M $MnSO_4$ and $10^{-5}$ M $CaCl_2$. The agar concentration was increased from 1.5 to 2%.

(2) Germination of spores was carried out in SPIZIZEN's minimal medium (SPIZIZEN 1958), supplemented with 0.02% casein hydrolysate (acid, vitamin free; Nutritional Biochemical Corp.); 50 $\mu$g per ml L-tryptophan, .0125 M L-alanine, 5 $\mu$g per ml thymine and 50 $\mu$g per ml 5-bromouracil. This germination medium will be referred to as SPG medium. Spores were germinated in 1 liter of SPG medium in Fernbach flasks on a reciprocal shaker at 37°C. Changes in the optical density during germination were followed using a Beckman Spectronic 20 at 600 m$\mu$. Incubation was terminated by addition of equal volumes of 2 × SPIZIZEN's salts and crushed ice.

*Preparation of competent cells and transformation:* The growth of cultures for production of competent cells was essentially that described by NESTER and LEDERBERG (1961). At the end of competence regimen sterile glycerol was added to make 40% v/v and the cells were frozen in a dry ice-ethanol bath. They were stored at —40°C until used.

The competent cells were exposed for 20 min at 37°C to 0.1 ml of the DNA fractions from the gradients (as described below) which had been diluted with 1 ml of 0.1 × SSC. The trans-

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TABLE 1

*Strains used as recipients*

| Strain | Marker | Mapping references | Source |
|---|---|---|---|
| SB-4 | *try-2* | NESTER, SCHAFER and LEDERBERG (1963) | NESTER via OKUBO |
| | *his-1* (L) | DUBNAU *et al.* (1967) | ..... |
| | *pur* (N) | unmapped | ..... |
| JB01–64 | *try-2* | NESTER, SCHAFER and LEDERBERG (1963) | OKUBO (OKUBO and ROMIG, 1966) |
| | *leu* (O) | isolated but unmapped by OKUBO | ..... |
| | *met* (O) | ..... | ..... |
| Mu8u5u2 | *his-1* (S) | YOSHIKAWA and SUEOKA (1963) | SUEOKA and YOSHIKAWA |
| | *met* (S) | YOSHIKAWA and SUEOKA (1963) | ..... |
| | *leu* (S) | YOSHIKAWA and SUEOKA (1963) | ..... |
| Mu8u5u5 | *thr* (S) | YOSHIKAWA and SUEOKA (1963) | SUEOKA and YOSHIKAWA |
| | *met* (S) | YOSHIKAWA and SUEOKA (1963) | ..... |
| | *met* (S) | YOSHIKAWA and SUEOKA (1963) | ..... |
| Mu8u5u16 | *ade* (S) | OISHI, YOSHIKAWA and SUEOKA (1964) | SUEOKA and YOSHIKAWA |
| | *met* (S) | OISHI, YOSHIKAWA and SUEOKA (1964) | ..... |
| | *leu* (S) | OISHI, YOSHIKAWA and SUEOKA (1964) | ..... |

formants were assayed on SPIZIZEN minimal agar with suitable supplements added, and counted after 48 hr incubation at 37°C. DNase and cell controls were included in each experiment.

*Preparation of spores.* Cells were incubated at least four days on TY agar at 37°C before the spores were harvested from the surface with sterile distilled water. The spores were washed with distilled water and incubated with 50 μg per ml lysozyme (=Muramidase, Worthington Biochemical Corp.) for 30 min at 37°C, washed in distilled water and heated at 80°C for 10 min. After another three washes in distilled water the spores were stored at 4°C until used.

*DNA preparations and CsCl density gradient centrifugation.* DNA was extracted essentially by the method of SAITO and MIURA (1963); 50 μg per ml protease-PK (Calbiochem.) was added after the lysozyme treatment to liberate DNA from protein.

Analytical density gradient centrifugation was performed in CsCl as described by MESELSON, STAHL and VINOGRAD (1957). The initial density was adjusted to 1.750 g per ml. Densitometric tracings of the UV absorption pattern were made with a Beckman analytrol. For calculating the percent DNA replicated the films were traced with a Joyce Lobel Densitometer and calculations made by weighing paper tracings of the area under the curves.

Preparative density gradient centrifugation was carried out in CsCl (initial density 1.750 g per ml) in a Spinco model L or L2 with a SW 39 rotor at 34,000 rpm for 72 hr at 25°C. Five drops per fraction were collected; and the refractive index was determined on alternate samples with a Zeiss Abbe refractometer. One ml of 0.01 × SSC ($1.5 \times 10^{-3}$ M NaCl, $1.5 \times 10^{-4}$ M Na citrate) was added to each fraction before the transforming activity was assayed. The original DNA or markers replicated once was calculated by the relationship:

$$\frac{\% \text{ Hybrid}}{\% \text{ Hybrid} + 2(\% \text{ Light})} \quad (1)$$

The original DNA or markers replicated twice was calculated by:

$$\frac{\% \text{ Heavy}}{\% \text{ Hybrid} + 2(\% \text{ Light})} \quad (2)$$

## EXPERIMENTS AND RESULTS

*DNA replication in 5-Bromouracil containing medium:* Spores of 168 *thy*⁻ were germinated in SPG medium and the optical density of the culture was followed during germination (Figure 1). An initial drop in optical density was observed followed by a rise after 120 min. When the culture was monitored for longer periods than shown in Figure 1 (200–300 min) a drop in optical density was observed, and the cells appeared to lyse. The significance of this drop in optical density during the later stages of growth in 5-BU containing medium will be the subject of another paper (BOICE, EBERLE, ROMIG and HUANG 1969). Samples were removed at various times indicated in Figure 1 and DNA was purified. These DNA samples were subjected to both analytical and preparative CsCl density gradient centrifugation. Figure 2 shows the results of the analytical study. The percent of the original DNA which had been replicated at least once and the percent which had been replicated twice were calculated and are shown in Figure 3A. About 18–20% of the DNA is of intermediate to hybrid density by 100 min. This remains constant until about 125 min, then a steady increase in DNA of hybrid density is observed. At a time when about 45–50% of the DNA has been replicated once, DNA of heavy density appears, indicating the onset of dichotomous replication. This is in agreement with the findings of OISHI, YOSHI-

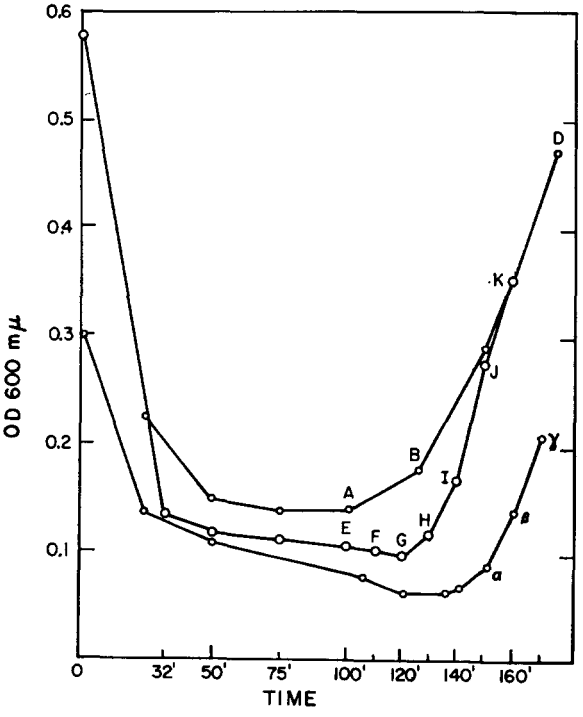


FIGURE 1.—Germination of *B. subtilis* spores in 5-BU containing medium. Optical density was followed in three separate experiments with A,B,D as one, E,F,G,H,I,J and K another, and $\alpha$, $\beta$, $\gamma$ the third. These letters also represent samples taken for DNA analyses.

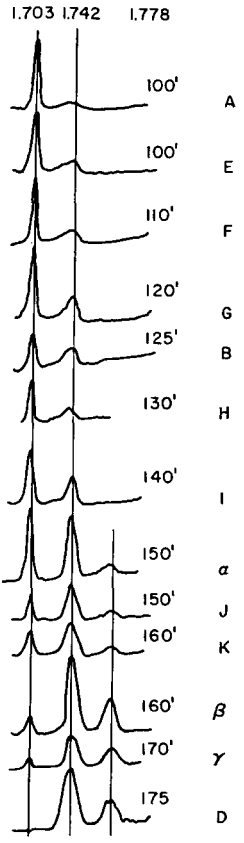


FIGURE 2.—Analytical isopycnotic density gradient analyses of DNA replication pattern. Density gradient centrifugation was made with a Beckman Model E ultracentrifuge equipped with a 12 mm cell having a Kel-F centerpiece. UV absorption at equilibrium (An-D rotor, 44,770 rpm, 25°C, 18–20 hrs) was photographed and the density traced by Beckman Analytrol and a Joyce Lobel Densitometer. Concentration of DNA was adjusted to about 6 μg and a CsCl isodensity of 1.750 g/cm$^3$ at the beginning of the centrifugation.

KAWA and SUEOKA (1964) for DNA replication during germination in 5-BU containing medium. The rates of replication for DNA replicated once and for DNA replicated twice appear to be different as judged from the slopes of the curves in Figure 3A.

*Marker replication in 5-BU containing medium.* The replication of some individual markers was followed during germination of 168 *thy*$^-$ in SPG medium. Samples were taken at times indicated in Figure 1. The DNA from these samples was subjected to CsCl density gradient centrifugation and the fractions were assayed for transforming capacity. Operationally, SUEOKA's mapping positions were assumed. One marker which maps near the origin of the chromosome [*his-1* (L)], one near the middle [*leu* (O)] and one near the terminus [*met* (O)] were chosen for this study. The rates of replication of these markers with time can be seen in Figure 3B and Figure 4. Very little replication of these markers

occurs before 120 min. However, 4–6% of the *his-1* (L) and 2–3% of the *pur* (N) marker are present in this early DNA of hybrid density as compared to 0.05-0.5% of the *leu* (O) and *met* (O) markers. At 210 min replication of the *his-1* (L) and *pur* (N) markers increases, followed by the *leu* (O) marker at 140 min and *met* (O) at 150 min. By 150 min 85% of the *his-1* (L) and *pur* (N) has been replicated once; by 175 min the replication of these markers appears to have leveled off at 90% above the original acitvity. Thus, approximately 10% of these markers failed to replicate by 175 min.

The degree of synchrony of replication in the population of germinating spores can be estimated by the rates of marker replication. It can be seen that 85-90% of the *his-1* (L) and *pur* (N) markers were replicated once before replication of the *met* (O) marker started. Thus, it is obvious from Figure 3B that not all chromosomes start replication at the same time, and that the range of starting times spans a period of about 30 min (120–150 min). By 175 min approximately 67% of the *met* (O) marker has been replicated. The residual transforming activity for the early markers observed in the unreplicated DNA at 175 min apparently is a small amount of the total DNA which may not replicate.

At, or shortly after, 140 min the onset of dichotomous replication of the *his-1* (L) and *pur* (N) markers begins, followed by *leu* (O) at 160 min and *met* (O)

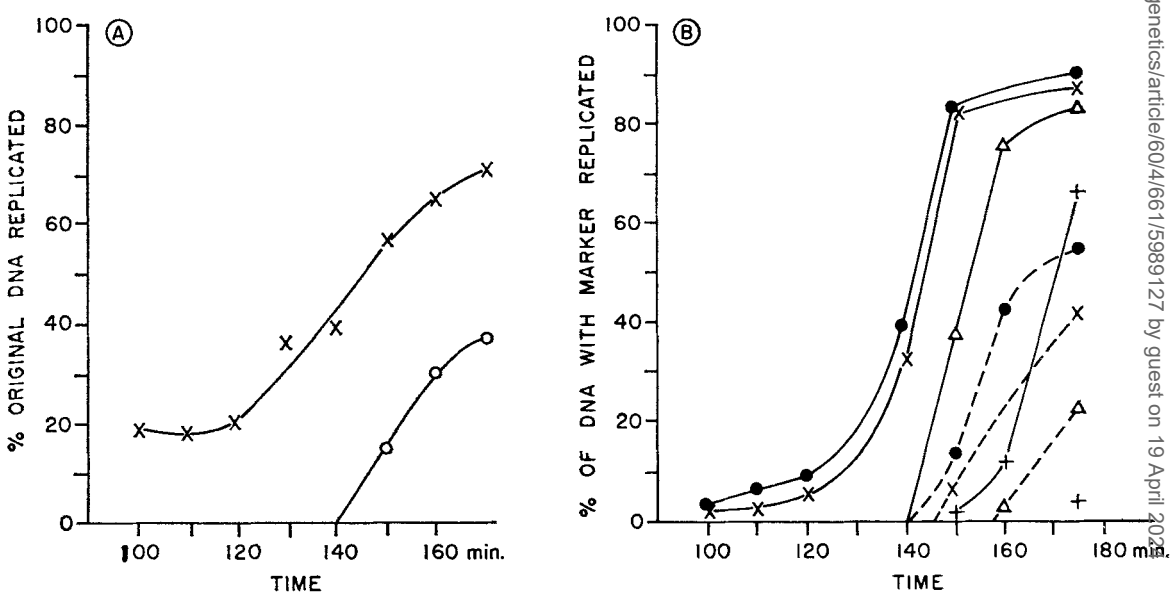


FIGURE 3.—Replication of DNA and markers during germination in 5-BU containing medium. (A) percent DNA replicated once ×××, samples taken from preparations in the order of A,F,G,H,I,J,K and γ for various points (see Figure 2). DNA replicated twice ○○○, samples taken from preparations J, K and γ. (B) percent DNA with marker replicated, solid line, replicated once; dotted line, replicated twice. *his-1* (L) ●●●; *pur* (N) ×××, *leu* (O) △△△, *met* (O) +++. Amount of DNA was calculated from densitometric tracings described in Figure 3 and from the transformation test in Figure 4.

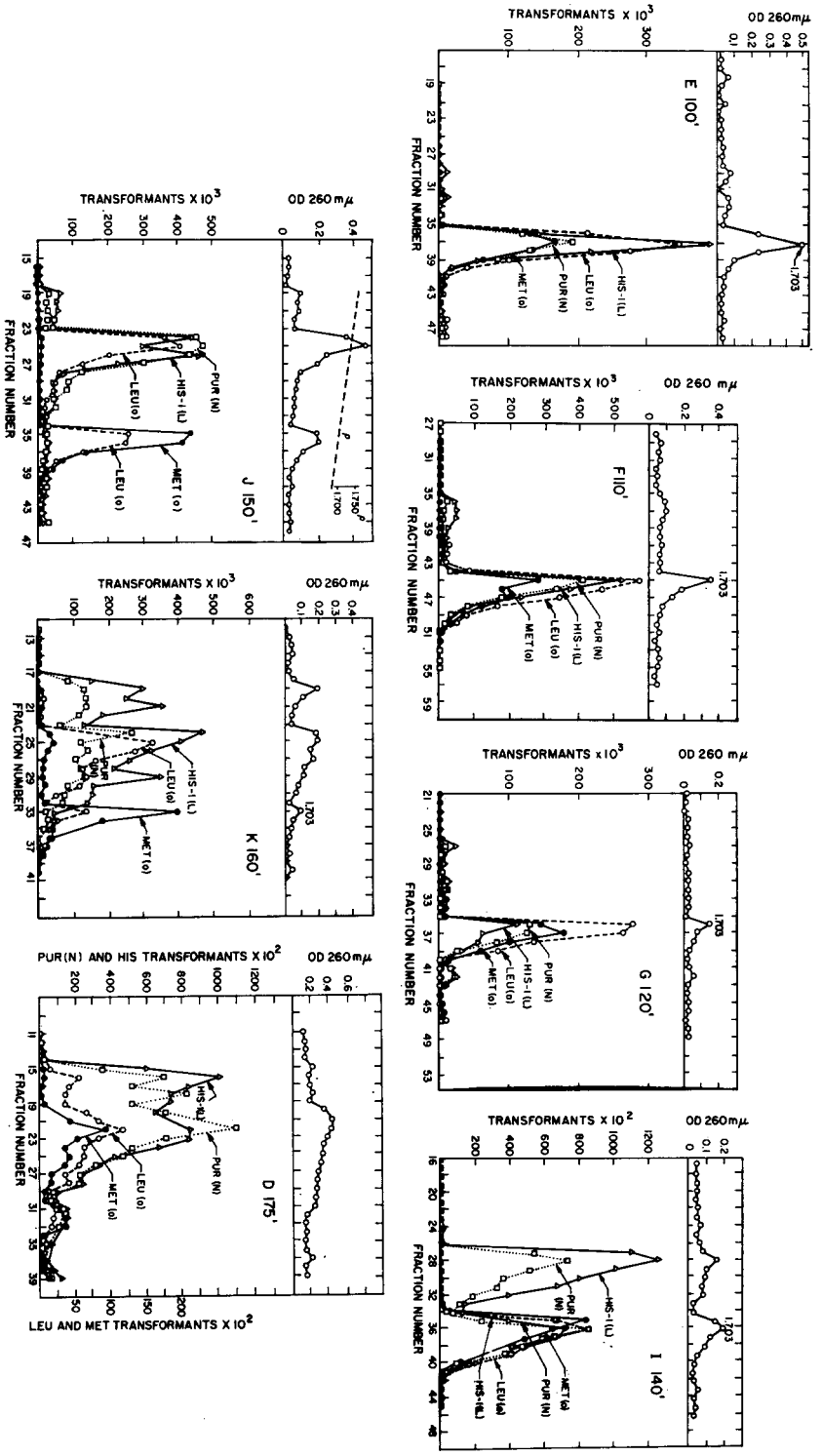


FIGURE 4.—Detailed synchro-transfer analysis for transformation of markers. See METHODS for details.

at 175 min. The rates of appearance of markers replicated twice appear to decrease as a function of their distance from the origin.

*Replication order of* his-1 *(L),* pur *(N),* leu *(O) and* met *(O) markers:* The distribution of markers in seven samples from two density transfer experiments (see the transformation profiles in Figure 4) is shown in Table 2. The number of transformants for each marker at the peaks of each sample is presented with the exception of results between the peak fractions (light and hybrid; hybrid and heavy). These fractions were excluded from the calculations because the origin of DNA molecules of intermediate density is uncertain; they may be partially replicated molecules, from the first or second replication or unreplicated molecules which have undergone repair processes (O'SULLIVAN and SUEOKA 1967).

The relative replication order of the four markers is consistent from 110 to 175 min after germination commences. Thus:

origin—*his-1* (L)—*pur* (N)—*leu* (O)—*met* (O)—terminus

TABLE 2

*Marker distribution in thymine to 5-bromouracil transfer experiment*

| Time of sample | Marker | Total number of transformants | Percent | | |
|---|---|---|---|---|---|
| | | | Light | Hybrid | Heavy |
| 100 | *his-1* (L) | 885 | 95.5 | 4.5 | 0 |
| E | *pur* (N) | 565 | 94.7 | 5.3 | 0 |
| | *leu* (O) | 1053 | 99.2 | 0.7 | 0 |
| | *met* (O) | 505 | 99.0 | 1.0 | 0 |
| 110 | *his-1* (L) | 1560 | 87.5 | 12.5 | 0 |
| F | *pur* (N) | 1155 | 94.8 | 5.2 | 0 |
| | *leu* (O) | 1810 | 100 | 0 | 0 |
| | *met* (O) | 935 | 100 | 0 | 0 |
| 120 | *his-1* (L) | 460 | 82.6 | 17.4 | 0 |
| G | *pur* (N) | 415 | 87.9 | 12.1 | 0 |
| | *leu* (O) | 800 | 96.2 | 3.8 | 0 |
| | *met* (O) | 505 | 99.0 | 1.0 | 0 |
| 140 | *his-1* (L) | 839 | 36.0 | 64.0 | 0 |
| I | *pur* (N) | 550 | 49.3 | 50.7 | 0 |
| | *leu* (O) | 327 | 99.4 | 0.6 | 0 |
| | *met* (O) | 276 | 99.3 | 0.7 | 0 |
| 150 | *his-1* (L) | 2000 | 8.2 | 78.5 | 13.2 |
| J | *pur* (N) | 2280 | 8.6 | 83.8 | 7.7 |
| | *leu* (O) | 2160 | 44.2 | 55.9 | 0 |
| | *met* (O) | 1275 | 96.1 | 3.9 | 0 |
| 160 | *his-1* (L) | 4235 | 5.5 | 59.3 | 35.3 |
| K | *pur* (N) | 2000 | 5.7 | 60.3 | 34.1 |
| | *leu* (O) | 1750 | 13.7 | 82.7 | 3.7 |
| | *met* (O) | 1000 | 78.0 | 22.0 | 0 |
| 175 | *his-1* (L) | 1045 | 5.4 | 56.7 | 37.9 |
| D | *pur* (N) | 932 | 6.3 | 62.2 | 31.5 |
| | *leu* (O) | 925 | 8.1 | 71.3 | 20.5 |
| | *met* (O) | 374 | 20.1 | 74.8 | 5.1 |

Although *pur* (N) is replicated very shortly after *his-1* (L) (Table 2), there is no evidence so far that they are linked. A cotransformation test (NESTER and LEDERBERG 1961) indicated that the percent cotransfer of *pur* (N) and *his-1* (L) is 0.6–1.9, which reflects an absence of linkage. DUBNAU, GOLDTHWAITE, SMITH and MARMUR (1967) could not detect linkage of *pur* (N) and *his-1* (L) by phage PBS1 transduction.

*Comparison of replicating order of* his-1 *(L)*, pur *(N)*, leu *(O) and* met *(O) with markers in* SUEOKA *strains:* The transforming activity in DNA fractions obtained at 140 and 150 min in one density transfer experiment was assayed using three recipient strains of SUEOKA and YOSHIKAWA. Only one fraction in the light and hybrid position was tested at each time; the marker distributions found are given in Table 3. The replication order of the markers from these two samples

TABLE 3

*A comparison of various markers in fractions of 140 and 150 minute samples*

| | | Total number of transformants | Percent light [I38] | Percent hybrid [I30] | |
|---|---|---|---|---|---|
| 140′ | | | | | |
| 1) | *ade-16* | 168 | 13.7 | 86.3 | |
| 2) | *his-1* (L) | 1294 | 38.2 (36.0) | 61.8 (64.0) | |
| 3) | *pur* (N) | 840 | 57.2 (49.3) | 42.8 (50.3) | |
| 4) | *thr* (S) | 489 | 79.6 | 20.4 | |
| 5) | *his-1* (S) | 2539 | 91.8 | 8.2 | |
| | *leu* (S)u2 | 2615 | 96.3 | 3.7 | |
| 6) | *leu* (S)u5 | 208 | 97.0 | 3.0 | |
| | *leu* (S)u16 | 222 | 100.0 | 0 | |
| 7) | *leu* (O) | 413 | 100 (99.4) | 0 (0.6) | |
| | *met* (S)u2 | 1900 | 97.7 | 2.3 | |
| 8) | *met* (S)u5 | 135 | 100 | 0 | |
| | *met* (S)u16 | 132 | 100 | 0 | |
| 9) | *met* (O) | 400 | 100 (99.3) | 0 (0.7) | |
| | | Total number of transformants | Percent light [J36] | Percent hybrid [J27] | Percent heavy |
| 150′ | | | | | |
| 1) | *ade-16* | 317 | 2.5 | 97.5 | ... |
| 2) | *his-1* (L) | 253 | 11.8 (8.3) | 88.2 (18.5) | (13.2) |
| 3) | *pur* (N) | 327 | 8.9 (8.5) | 91.8 (83.8) | (7.7) |
| 4) | *thr* (S) | 843 | 14.0 | 86.0 | ... |
| 5) | *his-1* (S) | 6407 | 42.0 | 58.0 | ... |
| | *leu* (S)u2 | 6391 | 52.9 | 47.1 | ... |
| 6) | *leu* (S)u16 | 232 | 61.7 | 38.3 | ... |
| | *leu* (S)u5 | 569 | 68.9 | 31.1 | ... |
| 7) | *leu* (O) | 374 | 66.0 (44.1) | 33.9 (55.9) | (0) |
| | *met* (S)u2 | 3871 | 91.3 | 8.7 | ... |
| 8) | *met* (S)u16 | 157 | 95.5 | 4.4 | ... |
| | *met* (S)u5 | 532 | 97.9 | 2.1 | ... |
| 9) | *met* (O) | 417 | 97.0 (96.1) | 3.0 (3.4) | (0) |

Values in ( ) are calculated from the whole peak.
I and J denote the origin of DNA sample.

is generally consistent and agrees with the replication order described above for *his-1* (L), *pur* (N), *leu* (O) and *met* (O). The relative order is: origin—*ade-16*—*his-1* (L)—*pur* (N)—*thr* (S)—*his-1* (S)—[*leu* (S) *leu* (O)]—[*met* (S) *met* (O)]—terminus. The other of the markers within the square brackets could not be determined from these samples. Both *leu* markers appear to be replicated at the same time. The *met* markers are replicated very late. No further genetic analysis has been done with these markers. However, *his-1* (L) and *his-1* (S) mapped differently, although DUBNAU, GOLDTHWAITE, SMITH and MARMUR (1967) assumed them to be identical.

*Mapping the* pur *(N) marker:* An attempt to map the *pur* (N) marker was made by the method of O'SULLIVAN and SUEOKA (1967). In mapping this marker we have assumed that: (1) the *his-1* (L) marker in our recipient strain (SB-4) is the same as the one mapped by O'SULLIVAN and SUEOKA in Mu8u5u5; (2) that the *met* (O) marker is at or near the *met* (S) marker = 1.0, where 1.0 represents the complete length of the genome; and (3) the *leu* (O) marker is near the *leu* (S) marker at 0.62. The comparison with the percent of the above markers in DNA of hybrid density in the 140 and 150 min samples (Table 3) indicates similar values for the *met* (O) and *met* (S) and for the *leu* (O) and *leu* (S) markers. Thus, this would support the above assumptions.

The values obtained for the *pur* (N) marker by the synchro-transfer method can be seen in Table 4. These values, calculated from the 120, 140 and 175 min samples, range between 0.1–.26 map units from the origin (*ade-16* = 0.00).

The average values of $\sigma$ for the various samples indicate that the most reliable values are those obtained from the 140 min sample. The other values should be considered qualitative (O'SULLIVAN and SUEOKA 1967). Thus, the lower values which range from 0.1 to 0.15 map units from the origin probably reflect the more accurate map distance for the *pur* (N) marker.

## DISCUSSION

The pattern of the replication of individual markers at the beginning of replication appears similar to the replication of total DNA. The significance of the early (100–200 min) DNA of intermediate to hybrid density is not clear (YOSHIKAWA 1965; WAKE 1967). The rates of replication of the four markers tested appear similar once replication of the markers commences during the first replication. During the dichotomous phase of replication the rate of markers replicated at the secondary forks appears to decrease as a function of their distance from the origin. This may be due to a general slowing down of replication and gradual degeneration of synchrony in 5-BU containing medium. It also may be a reflection of the transition of some of the chromosomes into the third round of replication where equation (2) is no longer valid. However, since the rate of total DNA replication does not decrease during the dichotomous phase, the slight decrease in the rates of markers replicated twice may be due to a decreased capacity of 5-BU containing DNA to transform.

The synchrony of germination can be followed by the rise in optical density.

TABLE 4

*Calculation of map position for* pur*(N) marker*

| Sampling time | Marker | Percent $X_{oo}$ | $X_{on}$ | $X_{nn}$ | $Ax$ | $tx$ | $\bar{x}$ | $X$ | Average value of $\sigma$ |
|---|---|---|---|---|---|---|---|---|---|
| | *his-1* (L) | 82.6 | 17.4 | .00 | .095 | 1.31 | —.9850 | .03* | 0.77 |
| 120′ | *pur* (N) | 87.9 | 12.1 | .00 | .064 | 1.52 | | .19 | |
| | *met* (O) | 99.0 | 1.0 | .00 | .005 | 2.56 | (first) | 1.0* | |
| | *his-1* (L) | 36.0 | 64.0 | .00 | .47 | .08 | .0016 | .03* | |
| 140′ | *pur* (N) | 49.3 | 50.7 | .00 | .34 | .41 | (first) | .15 | 0.36 |
| | *met* (O) | 99.3 | 0.7 | .00 | .0036 | 2.81 | | 1.00* | |
| | *his-1* (L) | 36.0 | 64.0 | 0.00 | .47 | .08 | .0123 | .03* | |
| 140′ | *pur* (N) | 49.3 | 50.0 | 0.00 | .34 | .41 | (first) | .10 | 0.22 |
| | *leu* (O) | 99.4 | 0.6 | 0.30 | .003 | 2.75 | | .62* | |
| | *his-1* (L) | 5.4 | 56.7 | 37.9 | .55 | —.23 | | .03* | |
| 175′ | *pur* (N) | 6.3 | 62.2 | 31.5 | .42 | .21 | .0983 | .21 | .053 |
| | *met* (O) | 20.1 | 74.8 | 5.1 | .044 | 1.71 | (second) | 1.0* | |
| | *his-1* (L) | 5.4 | 56.7 | 37.9 | .55 | —.13 | | .03* | |
| 175′ | *pur* (N) | 6.3 | 62.2 | 31.9 | .42 | .21 | .1182 | .26 | 0.68 |
| | *leu* (O) | 8.1 | 71.3 | 20.5 | .23 | .74 | (second) | .62* | |

Values with *, notations and calculations are based on O'SULLIVAN and SUEOKA (1967). $X_{oo}$, $X_{on}$ and $X_{nn}$ denote fractional amounts of transforming activity of marker being examined in non-replicated (oo), once replicated (on) and twice replicated (nn) peaks in CsCl density gradients. $A_x$ denotes fraction of chromosomes whose replication point is distal to marker being examined. $X$ is the relative position of the marker on the chromosome; $t_x = \frac{x - \bar{x}}{\sigma}$; and $\bar{x}$ is mean position of $x$.
See Text for details.

The synchrony of DNA replication can be judged by the rates of marker replication. The time required for 85–90% of the *his-1* (L) marker to be replicated is approximately 30 min. The time required for the onset of replication of the *met* (O) marker is 30–35 min after the onset of replication of the *his-1* (L) marker.

The mapping of the *pur* (N) marker by synchro-density-transfer yields values of 0.10–0.26 units from the origin (*ade-16* = 0.00). The validity of this assignment to the *pur* (N) marker is dependent on the assignment of known values to the *his-1* (L), *leu* (O) and *met* (O) markers. The equivalence of the *leu* (S) and *leu* (O) and the *met* (S) and *met* (O) markers has not been determined under optimal conditions since only two fractions each from two samples were used to determine these equivalences. Thus, the above map assignment to the *pur* (N) marker must be made with these reservations.

It is noteworthy that DUBNAU, GOLDTHWAITE, SMITH and MARMUR (1967) using the transduction technique have mapped two purine markers, *pur* A16 and *pur* B6. *Pur* A16 corresponds to *ade-16* of SUEOKA, and *pur* B6 may be equivalent to the *pur* (N) studied here. Independent studies by us (unpublished)

and others (see OISHI and SUEOKA 1965, and DUBNAU, GOLDTHWAITE, SMITH and MARMUR 1967) have shown that some of the ribosomal RNA cistrons are located between these genes.

We wish to thank MISSES PAT MOCKUS, KAY MCGUIRE and MRS. SANDRA ANTOINE for their assistance during various phases of these studies.

## SUMMARY

DNA replication in germinating *Bacillus subtilis* has been studied with the synchro-transfer method using 5-Bromouracil as a density label. It is shown that semi-conservatively replicating DNA can be realized both in the amount of heavy label as well as by the appearance of genetic markers in transformation experiments. Results from this study affirm the replication sequence as:

origin—*ade-16*—*his-1* (L)—*pur* (N)—*thr* (S)—*his-1* (S)—[*leu* (S) *leu* (O)]—[*met* (S) *met* (O)]—terminus.

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