THE *BACILLUS SUBTILIS* GENOME:
REPLICATION-ORDER AND MAP-POSITION DISCREPANCIES—
EVIDENCE FOR A SECOND ORIGIN

JAMES C. COPELAND

*Department of Microbiology, College of Biological Sciences,
Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210*

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ABSTRACT

Replication order to map position discrepancies have been documented in *B. subtilis*. The discrepancies were found to occur whenever the genome of *B. subtilis* was replicated under a variety of physiological conditions and in both 168 and W23 strains. The earliest replicating marker involved in these discrepancies was *thr* versus *purB* and *aroA* versus *recA*. A detailed linkage analysis of the *ura* to *argA* region was consistent with its being a continuous linkage group. This led to the conclusion that an origin for new starts at replication exists between *recA* and *aroA*.

THERE are three ways in which the genome of *B. subtilis* can be studied: by conventional linkage analysis using transformation (SPIZIZEN 1958) and transduction (TAKAHASHI 1963); by replication mapping using marker frequency analysis (YOSHIKAWA and SUEOKA 1963a) and density transfer experiments (YOSHIKAWA and SUEOKA 1963b) both in conjunction with transformation; and by physical means such as autoradiography (DENNIS and WAKE 1968). Accumulated linkage analysis data have indicated three unequal linkage groups (DUBNAU 1970; YOUNG and WILSON 1972). The first includes genetic markers linked to *purA*, the second consists of markers linked to *purB*, and the third linkage group is a combined form of two earlier linkage groups. The third linkage group is the largest of the three and includes genetic markers ranging from *thr*-*citK*.

Replication mapping has been generally consistent with those markers examined as being replicated in a sequential order (YOSHIKAWA and SUEOKA 1963a, 1963b). An origin proximal to *purA* and a terminus distal to the *metB* marker for replication has been defined from these studies. Almost all other markers have been found to replicate in a sequential order consistent with linkage-map order. When a comprehensive analysis was done employing a large number of genetic markers, replication discrepancies were noted which correspond to those discussed in this paper (O'SULLIVAN and SUEOKA 1967). It should be emphasized that a limited number of genetic markers has been examined in any one study by these techniques.

Early autoradiographic evidence did not show a grain pattern which could

FIGURE 1.—A composite genetic map for *B. subtilis* representing a current model for chromosome organization (adopted from DUBNAU 1970; YOUNG and WILSON 1972).

account for all of the DNA in the genome of *B. subtilis* (DENNIS and WAKE 1968). But loops were found internally on linear molecules, and this evidence suggested the possibility that the origin and terminus of replication were physically linked, analogous to the Cairns model for the chromosome of *Escherichia coli* (CAIRNS 1963).

Taken together, the genetic linkage analysis, replication order mapping, and autoradiographic data have led to the current model for the organization of the *B. subtilis* genome (Figure 1). This model presents the genome as being organized as a single linkage group, which replicated from a single origin in a unidirectional, sequential order. The origin and terminus were expected to be linked, which would make for a closed loop structure (DENNIS and WAKE 1968; SUEOKA and QUINN 1968; RAMAREDDY and REITER 1970; YOSHIKAWA 1970). In other words, this model presents the genome of *B. subtilis* as a single, circular chromosome which replicates unidirectionally.

More recently, autoradiographic data have been presented which showed that the *B. subtilis* chromosome is capable of bidirectional replication over at least 20% of its length (GYURASITS and WAKE 1973), if the genome exists as a single chromosome. This finding requires a significant modification of the earlier unidirectional model for the *B. subtilis* chromosome. Also, a recent study of additional markers near the known origin is consistent with bidirectional replication (HARA and YOSHIKAWA 1973; YAMAGUCHI and YOSHIKAWA 1973; COPELAND and HARRIS, unpublished observations). The results of this report will show a significant discrepancy in the replication order of markers in the proposed third linkage group. This and a re-examination of linkage among markers in this third group show that an origin for starts at replication exists in this linkage group.

MATERIALS AND METHODS

Bacterial strains: Table 1 lists the strains and cell lines of *B. subtilis* used in this study. It includes their sources and genotypes.

TABLE 1
List of *B. subtilis* strains

Strain*	Other designation	Genotype†	Source
BC26		<i>phe-12 argA3 ery-1</i>	D. DUBNAU
BC29	168-2	<i>leu-2 trp-2</i>	J. PENE
BC34		<i>ura</i>	D. DUBNAU
BC35	Mu8u5u6	<i>purB6 leu-8 metB5</i>	N. SUEOKA
BC36	Mu8u5u5	<i>thr-5 leu-8 metB5</i>	N. SUEOKA
BC39		<i>argC4 leu-2 trp-2</i>	J. MARMUR
BC50		<i>purA16 leu-8 nia-38 metB5</i>	
BC51		<i>leu-8 lys-21 metB5</i>	
BC67		<i>cysB his-1 trp-2</i>	E. LEDERBERG
BC71	GSY 1006	<i>his-25 trp-16 recA</i>	C. ANAGNOSTOPOULOS
BC73	PB 3046	<i>fur</i>	M. POLSINELLI
BC86		<i>purA16 argA3 metB5</i>	
BC87		<i>purA16 ura metB5</i>	
BC88		<i>argC leu-8 metB5</i>	
BC92		<i>purA16 argA ile</i>	
BC100		<i>purA16 leu-8 metB5</i>	N. SUEOKA
BC115	WB2281B	<i>aroA</i>	E. NESTER
BC200		<i>leu-8 metB thyA thyB</i>	
BCW503	SB12	<i>thr his leu</i>	E. NESTER

* BC designates 168 strains and BCW designates W23 strains.

† Gene symbols indicate mutations leading to requirements for/or resistance to the following: *arg* (arginine), *aro* (shikimic acid for aromatic amino acids), *cys* (cysteine), *leu* (leucine), *lys* (lysine), *met* (methionine), *nia* (niacin), *phe* (phenylalanine), *pur* (adenine or guanine), *thr* (threonine), *thy* (thymine), *trp* (tryptophan) and *ura* (uracil). The symbol *rec* indicates a mutation that effects recombination ability.

Media and growth conditions: The minimal salts composition has already been given (COPELAND and MARMUR 1968). Complete liquid minimal medium contained, in addition to the salts, glucose (0.5 percent w/v) and required growth factors at 50 µg/ml, unless otherwise noted. Solid minimal medium contained the same ingredients as the liquid medium and 2% (w/v) agar plus asparagine and glutamic acid each at 50 µg per ml. Selective medium used to isolate specific recombinants was made on solid minimal medium lacking the particular growth factor required by the recipient cell line and for which recombinants were desired. The donor genetic material, providing the requisite genetic information to make the particular growth factor, was supplied via transformation or transduction.

Complex medium was Brain-Heart infusion medium (BHI, Difco, Detroit, Michigan). In all cases it was supplemented with thymine and adenine each at 50 µg/ml. Solid medium contained agar at 2% (w/v) concentration.

Media in the procedure used for making competent cells remained essentially as described (ERICKSON and COPELAND 1972). Also, fully deuterated medium used in the density transfer experiments has been described (COPELAND 1971a). The liquid minimal salts solution served as diluent when needed.

Growth conditions will be described for the specific experiment in the text. Where indicated, cells in balanced growth were carefully prepared using the procedure already described (COPELAND 1969). The method used to starve cells for a required amino acid has been given (COPELAND 1969). Unless otherwise stated all incubations were at 37°. Solid minimal medium plates were incubated for 2 days before colonies were counted. Optical densities of cultures were measured on a Klett-Summerson colorimeter using a blue filter (No. 42) for minimal medium and a red filter (No. 64) for complex medium.

Transformation and competent cells: The procedures for preparing and freezing competent

cells remained essentially the same (ERICKSON and COPELAND 1972). Competent cells were concentrated 10 $\times$ in SpII plus 10% glycerol (v/v) and quick-frozen in a dry ice ethanol bath. They were stored in a liquid nitrogen refrigerator. For use, the competent cells were quickly thawed and resuspended to the original concentration by diluting with fresh SpII medium lacking glycerol. Samples of DNA, in a volume of 0.1 ml, were mixed with 0.9 ml of competent cells. They were incubated with shaking at 37° for 30 minutes. The mixture was then iced, diluted when necessary, and plated for transformants on selective media.

Transduction with PBS1: Methods used for producing transducing phage lysates and for carrying out transduction have been described (COPELAND and MARMUR 1968). The only change introduced was to add glycerol (10% v/v) to the DNase treated lysate. Aliquots of the lysate were distributed to tubes, frozen in a dry ice ethanol bath, and stored in a liquid nitrogen refrigerator. For use, the transducing lysate was quickly thawed and a few drops of chloroform were added and mixed with the lysate. The lysate was then incubated at room temperature for about 30 minutes before a sample of phage was taken for addition to cells.

DNA isolation and DNA measurements: The protocol for DNA isolation, for the density transfer experiment and for marker frequency analysis, was described in detail (COPELAND and MARMUR 1968; ERICKSON and COPELAND 1972). Spore DNA was isolated using the method of TAKAHASHI (1968). Measurement of the amounts of DNA present was made chemically as already described (COPELAND 1969).

Density transfer experiments: Density transfer experiments using germinating spores have been described (COPELAND and MARMUR 1968). The protocol for density transfer following amino acid starvation was presented before (COPELAND 1971a). Those methods used in density transfer of stationary phase strain W23 cultures from deuterated to hydrated medium were also described (COPELAND 1971a).

Marker frequency analysis: Marker frequency analysis was performed on DNA samples also used in density transfer experiments as well as on DNA samples not involved in density transfer experiments. The details are provided in the text. Methods for marker frequency analysis were presented (COPELAND 1971b).

RESULTS

Discrepancy between replication order and map position: A current model of how the *B. subtilis* genome is organized has it as a single chromosome. This chromosome is thought to be circular and to replicate unidirectionally and sequentially from an origin located near *purA*. A representation of a composite linkage and replication map is shown in Figure 1 (taken from DUBNAU 1970 and from YOUNG and WILSON 1972).

According to this model each marker is expected to replicate in a predictable sequence with those markers closest to the origin replicating first and distal markers replicating later. In density transfer experiments in which DNA replication was restarted following leucine starvation (COPELAND 1969), certain genetic markers were replicated ahead of more proximal markers. This was observed by both 168 (COPELAND 1969) and W23 (COPELAND 1971a) strains of *B. subtilis*. The genetic markers found to replicate out of order were *hisA* versus *purB* and *lys* versus *argC* (Figure 2). A number of possibilities existed to explain these observations. This discrepancy might be explained by preferred stopping sites in these regions where replication forks came to rest in lieu of reaching the terminus, since replication did not terminate during leucine starvation. Another possibility was that origins existed in these regions where new starts at replication could be made. Also, trivial explanations could account for replication order

FIGURE 2.—Transformation assay of a gradient taken when net DNA synthesized was 55% in BC200 cells. The cells were previously starved of leucine for 150 min in H_2O medium before transfer to D_2O medium. LL indicates peak of light fraction, LH that for the hybrid fraction, and HH for the heavy fraction.

discrepancies such as artifacts of the density transfer experiment and/or the markers involved could have simply been mismapped. Together with the model for unidirectional and sequential replication of a single, circular chromosome, these observations seemed most consistent with the preferred stopping site model (COPELAND 1969). The studies reported here prove this is not true:

Discrepancy in replication sequence and map position—not a function of amino acid starvation: The first hypothesis to be tested was that these replication order discrepancies were strictly a function of amino acid starvation, specifically starvation for leucine. This was done by studying replication order in germinating spores. Others (WAKE 1963; OISHI, YOSHIKAWA and SUEOKA 1964; O'SULLIVAN and SUEOKA 1967) have shown that replication in germinating spores is highly synchronized from the origin proximal to *purA*. Spores of strain W23 were harvested from hydrated medium and germinated in a deuterated medium. Samples were taken at different times during outgrowth and the net amount of DNA synthesized was determined by chemical means. Appropriate samples were chosen for DNA isolation and fractionation on a preparative cesium chloride density gradient. The fractionated samples were assayed by transformation activity for a large number of genetic markers. The results are shown in

FIGURE 3.—Transformation assay of a gradient taken when net DNA synthesized was 38% in BCW503 germinated spores. The spores were harvested from a H_2O medium and germinated in a D_2O medium. LL indicates peak of light fraction, HL that for the hybrid fraction, and HH for the heavy fraction.

FIGURE 4.—Transformation assay of a gradient taken when net DNA synthesized was 70% in BCW503 germinated spores. Conditions as in Figure 3.

Figure 3 and 4 and Table 2. The sample used for the first gradient was taken after a net increase in DNA of 38% (Figure 3), and it showed highly synchronized replication starting from the *purA* site. But note that *cysB* and *argA* were already replicating out of their expected order, 55% first-round replication for *cysB* versus 47% for the more proximal marker *thr*, and 10% first-round replication for *argA* versus 2% for the more proximal marker *ura* (Table 2). Second-round replication was quite advanced in this gradient, as evidenced by the *purA* marker; 72% of this marker has replicated two or more times. This second-round replication activity or dichotomy was to be expected, since these spores were germinated in a nutritionally rich deuterated medium. In this gradient (Figure 3 and Table 2) it can be seen that *cysB* has also replicated to a greater extent for the second round than has its proximal neighbor *thr*, 7% versus 3%, respectively.

Continued DNA replication during outgrowth of these germinated spores was followed in the second gradient (Figure 4 and Table 2), at which time there was a net increase in DNA synthesized of 70%. At this time both *cysB* and *argA* have continued to replicate ahead of their most proximal neighboring markers on the genetic map. In this latter sample, the markers replicated out of order extended to the next marker distal to *cysB* and *argA* (Table 2). For example, *argC* had replicated 76% in the first round compared to 67% for *thr* and 82% for *cysB*. Also *nia* had replicated 43% in the first round compared to 24% for *ura* and 69% for *argA*. In other words, the replication out of order continues to the next neighboring marker. It is interesting that for both sites the direction of this replication is the same on the current genetic replication map (Figure 1), i.e., from origin toward the terminus.

TABLE 2

Replication of genetic markers in BCW503 during spore germination

Genetic marker	Total colonies per gradient	After 38% increase in DNA		Total colonies per gradient	After 70% increase in DNA	
		Percent replicated* One or more times	Two or more times		Percent replicated One or more times	Two or more times
<i>purA16</i>	1140	88	72	2220	94	91
<i>purB6</i>	830	72	8	1060	69	37
<i>thr</i>	2320	47	3	1420	67	20
<i>cysB</i>	330	55	7	230	82	59
<i>argC</i>	1320	12	—	2410	76	22
<i>ura</i>	860	2	—	1010	24	1
<i>argA</i>	950	10	1	2090	69	27
<i>nia</i>	1760	3	—	1180	43	9
<i>metB</i>	830	1	1	870	13	1

* Percent replication is determined from the relationship $(LH - HH)/2 + HH/2 \div (LL + (LH - HH)/2 + HH/2) \times 100$ for percent marker activity that has replicated one or more times. The numerator becomes $(LH - HH)/2$ for percent marker activity that has replicated only once. The difference between them yields the percent marker activity that has replicated two or more times. *LL* is equivalent to transforming activity in the light fraction, *LH* with that in the hybrid fraction, and *HH* with that in the heavy fraction, each expressed as percent of total transforming activity in the gradient.

The exact extent of this replication order to map position discrepancy can be better seen in the comparative plots of Figures 5 and 6. The *cysB* marker has replicated to a greater extent than *purB*, a marker thought to more proximal in replication order (Figure 5). The *argA* marker has clearly replicated ahead of *ura*, a marker assumed to be a proximal neighbor (Figure 6).

Still another experiment was done to determine whether this discrepancy between order of replication and map position occurred under different physiological conditions. YOSHIKAWA and SUEOKA (1963a) have demonstrated that replication is ordered when one transfers stationary phase cells of *B. subtilis* strain W23 to fresh medium to restart growth. This experiment was performed using W23 cells grown to stationary phase in fully deuterated medium. The cells were then washed free of the deuterated medium and resuspended with complex hydrated medium. Samples were taken at different times during growth, and the net amount of DNA synthesized was determined. Appropriate samples were selected for fractionation on cesium chloride preparative gradients. The collected fractions were then assayed for transforming activities. The results are displayed in Figures 7 and 8 and in Table 3.

The sample for the first gradient was taken when the net increase in DNA synthesized was 34% (Figure 7). The synchrony of replication in these cells is very high, as shown by the extent of replication at the *purA* (70%) and *purB* (30%) sites compared to the low amount of replication activity at all other sites (less than 5%). As expected in this medium, replication is proceeding dichotomously, as shown by the second round replication activity at the *purA* site (21%).

FIGURE 5.—Detail of transformation assay of gradients in Figures 3 and 4 comparing *purA*, *purB* and *cysB* markers. Panel A shows sample taken after net DNA synthesized was 38% and panel B after 70%. Conditions as in Figure 3.

FIGURE 6.—Detail of transformation assay of gradients in Figures 3 and 4 comparing *ura*, *argA* and *metB* markers. Panel A shows sample taken after net DNA synthesized was 38% and panel B after 70%. Conditions as in Figure 3.

FIGURE 7.—Transformation assay of a gradient taken when net DNA synthesized was 34% in BCW503 cells first grown to stationary phase in D_2O medium and then transferred to an H_2O medium. LL indicated peak of light fraction, HL that of the hybrid fraction, and HH of the heavy fraction.

FIGURE 8.—Transformation assay of a gradient taken when net DNA synthesized was 63% in BCW503 stationary phase cells transferred from D₂O medium to H₂O medium. Conditions as in Figure 7.

TABLE 3

Replication of genetic markers in BCW503 grown from stationary phase

Genetic marker	Total colonies per gradient	After 34% increase in DNA Percent replicated*		Total colonies per gradient	After 63% increase in DNA Percent replicated	
		One or more times	Two or more times		One or more times	Two or more times
<i>purA16</i>	1400	70	21	2290	84	28
<i>purB6</i>	1490	30	5	1520	72	22
<i>thr</i>	1330	4	—	2000	65	5
<i>argC</i>	1350	4	—	2160	51	11
<i>ura</i>	620	4	—	1040	25	3
<i>argA</i>	550	2	—	2230	54	11
<i>lys</i>	1420	3	—	1360	18	2
<i>metB</i>	600	4	—	3140	10	2

* See footnote to Table 2.

The second gradient was sampled after a net increase in DNA synthesized of 63%. At this time *argA* showed replication activity greater than its proximal neighbor *ura*, 54% first round activity *versus* 25%. Markers in the *purB* to *argC* region do not show the discrepancy between order of replication and map position for the first round, but note that *argC* is showing significant second-round activity (11%) compared to its proximal neighbor *thr* (5%). The same is true for *argA* (11%) *versus* *ura* (3%) as regards second-round replication activity. A detail of the *argA* replication profile is shown in Figure 9. Here it is obvious that *argA* has replicated out of order relative to its map position.

FIGURE 9.—Detail of transformation assay of gradients in Figures 7 and 8 comparing *argC*, *ura* and *argA* markers. Panel A shows sample taken when net DNA synthesized was 34% and panel B after 63%. Conditions as in Figure 7.

Results of these experiments show that this discrepancy between order of replication and map position is not restricted to regrowth of leucine-starved cells, but can be demonstrated during the outgrowth of germinated spores and during regrowth of stationary phase cells of strain W23. The results with the stationary phase cells also showed that this discrepancy is not an artifact of growth in the deuterated medium, since replication in these cells was in a hydrated medium. This point will be more clearly made in the next section. The discrepancy between order of replication and map position is most readily and consistently demonstrated for the *ura-argA* region. The variable observations in the *purB-argC* region are unexplained.

Discrepancy between replication order and map position demonstrated by marker frequency analysis: The next hypothesis to be tested was whether this discrepancy between order of replication and map position was possibly an artifact of the density transfer experiment, which involved the use of deuterated media. This was accomplished by use of marker frequency analysis, which provides an independent measure of marker replication without resorting to use of any density label. The density transfer experiment is a much more sensitive measure of replication activity than marker frequency analysis. Density transfer experiment is less prone to external errors, such as day-to-day variations in media and competent cells and normalization with standard DNA, and internal errors such as designation of a reference terminal marker, than is the marker frequency analysis. Nevertheless, the differences observed in order of replication by the density transfer experiment were of sufficient magnitude that significant differences should be observable with marker frequency analysis.

The first marker frequency analyses were done on DNA samples used in the density transfer experiments for the spore germination and outgrowth and for the stationary phase restart experiments. Since a limited sample of DNA remained, the analysis was performed on the *argA/metB* pair versus the *ura/metB* pair (Table 4). For germinated spores, when the first sample was taken (plus 38% DNA), there was no significant difference between these ratios, although the density transfer gradient (Figure 3) showed *argA* to be replicated to a greater extent than *ura* (10% versus 2%). By the time the second sample was taken (plus 70% DNA) a significant difference was observed between the *argA/metB* ratio (1.63) and the *ura/metB* ratio (1.12), in agreement with the replication order observed in the gradient (Figure 4).

A marker frequency analysis was performed on the DNA sample used to generate the gradient shown in Figure 8 of the stationary phase restart experiment. The results (Table 4) show a larger ratio for *argA/metB* compared to *ura/metB*, but the difference is statistically insignificant. The gradient obtained from this sample of DNA had shown *argA* to have replicated ahead of *ura* (Figure 8).

Another comprehensive marker frequency analysis was performed on stationary phase W23 cells analogous to the experiment done for density transfer. However, in the experiment for marker frequency analysis, only hydrated medium was used throughout, and it was nutritionally a minimal medium. Samples were taken at intervals during regrowth of the stationary phase cells. The net amount of DNA synthesized was determined, and marker frequency analysis was done on a number of strategically located markers (Table 5). In the DNA sampled from stationary phase cells, the marker frequency ratio of all markers relative to *metB* was not significantly different from 1.0 except for *argC*. This would be expected if the genome were not replicating, since all markers would be present in equal numbers. This result is consistent with the density transfer experiment and with the experiments of others (YOSHIKAWA and SEUOKA 1963a). Upon restart of growth (plus 38% DNA) the marker frequency ratio increased for the first three most proximal markers *purA*, *purB* and *thr* (positions as shown in Figure 1). The marker frequency ratio for *thr* was greater than *purB* but the difference is insignificant. Continued growth (plus 59% DNA) showed an increase in marker frequency ratio at the *purA* site. The *thr/metB* ratio was

TABLE 4

Marker frequency analysis on DNA samples used in density transfer experiments*

Genetic markers	BCW503 spore germination†						BCW503 stationary‡		
	N	+ 38% DNA X̄	SX̄	N	+ 70% DNA X̄	SX̄	Phase restart + 63% DNA N	X̄	SX̄
<i>ura/metB</i>	4	0.99	0.08	4	1.12	0.06	8	1.21	0.05
<i>argA/metB</i>	4	0.90	0.08	4	1.63§	0.08	9	1.31	0.16

* All marker frequency ratios were normalized with spore DNA.

† DNA samples were the same as those used to obtain the gradients in Figures 3 and 4.

‡ DNA sample was the same as that used to obtain the gradient in Figure 8.

§ The *argA/metB* ratio is significantly greater than the *ura/metB* ratio for this sample at the 5% level of significance.

TABLE 5

*BCW503 marker frequency analysis of stationary phase restart ($H_2O \rightarrow H_2O$)**

Genetic marker ratio relative to <i>metB</i>	Stationary phase			+38% DNA			+51% DNA			+59% DNA		
	N	$\bar{X}$	S $\bar{x}$	N	$\bar{X}$	S $\bar{x}$	N	$\bar{X}$	S $\bar{x}$	N	$\bar{X}$	S $\bar{x}$
<i>purA</i>	4	0.90	0.06	4	1.27	0.10	4	1.24	0.10	4	1.52	0.14
<i>purB</i>	8	1.04	0.05	6	1.16	0.06	6	1.19	0.09	8	1.13	0.04
<i>thr</i>	9	1.00	0.05	6	1.26	0.07	6	1.27	0.05	6	1.32†	0.03
<i>argC</i>	7	1.13‡	0.03	4	1.08	0.04	4	1.28	0.06	7	1.24	0.02
<i>ura</i>	3	1.01	0.04	3	1.07	0.03	3	1.04	0.01	3	0.98	0.06
<i>argA</i>	11	0.96	0.03	6	0.98	0.04	6	1.01	0.04	11	1.05	0.09
<i>nia</i>	7	1.20	0.09	7	1.16	0.10	7	1.18	0.07	7	1.21	0.08
<i>lys</i>	4	1.20	0.24	4	1.12	0.03	4	1.12	0.21	4	1.04	0.10

* All marker frequency ratios were normalized with spore DNA.

† The *thr* ratio is significantly greater than the *purB* ratio at the 5% level of significance in this sample.‡ The *argC* is significantly greater than 1.00 at the 5% level of significance.

significantly greater than the *purB/metB* ratio in this sample. Throughout this analysis the *nia/metB* ratio remained above 1.0 but was never significantly greater than its most proximal neighbor, *arg/metB*. These results are consistent with there being equal copies of genetic markers in stationary phase cells and with a sequential increase in relative numbers of copies among markers that are proximally located. During this replication, the *thr* marker exceeded the number of copies of its proximal neighbor *purB*. In other words, it had replicated out of order when compared to its map position.

Thus, the marker frequency analysis has confirmed the results observed with density transfer experiments. Markers found to have replicated out of order in a gradient also have replicated out of order by marker frequency analysis in cells grown from stationary phase. These results show that the discrepancy between order of replication and map position is not an artifact of the density transfer experiment. They are compatible with there being additional origins for starts at replication in these regions or with the organization of the genome being different from the current model or both.

Detailed genetic analysis of the ura-argA region: One remaining point that needed to be examined, in order to validate the observed order in replication, was the proper location of the markers used in this study. Are the genetic markers located as indicated in the current map for *B. subtilis* or have they been mis-mapped? Even though mismapping is a highly unlikely possibility, it deserves re-examination, since such finding would easily explain the apparent disorder in marker replication.

The two regions where discrepancies have been observed are *thr-argC* and *argA-nia*. The earliest replicating markers involved in replicating out of order are *thr* and *argA*, respectively. It may be more than coincidence that these markers reside at locations where linkage discontinuities exist. For example, *thr* has not been found linked to markers that replicate ahead of it, namely the *purB* group.

In fact, the location of *purB* on the genetic map in the current model (Figure 1) is based solely on its replication order. Also *ura* and *argA* markers have been reported linked and other markers have been found between them, but the degree of linkage is reportedly very low and has not been documented in the literature (DUBNAU 1970; YOUNG and WILSON 1972; HOCH, personal communication). Since the *ura* and *argA* regions are considered genetically linked (see Figure 1). I decided to re-examine the linkage relationships of those markers used in this study and also their replication order.

The linkage associations were determined by two-factor crosses using the generalized transducing phage PBS1. The results are presented in Table 6. The first four crosses were done to establish the locations of the markers of interest relative to other genetic markers in this region. For example, *ura* was shown to be linked to *recA*, which in turn was closely linked to *fur* (crosses 1 and 2 of Table 6). Also, *argA* was shown to be linked to *leu* and also closely linked to *aroA* (crosses 3 and 4 of Table 6). Note the ease with which these linkage relationships are demonstrated.

When crosses were done to test for linkage between *ura* and *argA*, much more effort was required to demonstrate even marginal linkage evidence. For example, no linkage was detected between *argA* and *recA* after a total of 773 recombinants were examined (crosses 5 and 6 of Table 6). When linkage between *recA* and *aroA* was examined only 1 positive isolate was found among 491 Rec⁺ recombinants (cross 7, Table 6), and again a single positive isolate was found among 473 Aro⁺ recombinants (cross 8, Table 6). This would give a 0.2% linkage association between *recA* and *aroA*, but I would like to stress that this linkage association rests on very slim evidence. Based on the number of transducing phage

TABLE 6

Two-factor analysis using PBS1 transducing phage

Cross Ø × cells	Linkage analysis	Selected* marker	Number of transductants†	Unselected marker	Number scored	Percent‡ linkage
1. BC71 × BC87	<i>ura-recA</i>	Ura ⁺	62	Rec ⁻ (MC ^s)	20	32%
2. BC73 × BC71	<i>recA-fur</i>	Rec ⁺ (MC ^r)	82	Fur ^s	72	88%
3. BC100 × BC86	<i>argA-leu</i>	Arg ⁺	202	Leu ⁻	50	25%
4. BC115 × BC86	<i>argA-aroA</i>	Arg ⁺	125	Aro ⁻	97	78%
5. BC71 × BC86	<i>argA-recA</i>	Arg ⁺	a) 92 b) 113 c) 97 d) 231 533	Rec ⁻ (MC ^s)	a) 0 b) 0 c) 0 d) 0 0	< 0.2%
6. BC92 × BC71	<i>recA-argA</i>	Rec ⁺ (MC ^r)	240	ArgA ⁻	0	< 0.4%
7. BC115 × BC71	<i>recA-aroA</i>	Rec ⁺ (MC ^r)	a) 115 b) 146 c) 230 491	Aro ⁻	a) 0 b) 0 c) 1 1	0.2%
8. BC71 × BC115	<i>aroA-recA</i>	Aro ⁺	a) 235 b) 238 473	Rec ⁻ (MC ^s)	a) 1 b) 0 1	< 0.2%

* Rec⁺ recombinants were selected by their resistance to 5 µg/ml mitomycin C (MC^r).

† Numbers listed in brackets are the results of individual crosses.

‡ Percent linkage was determined from pooled results where there was more than one cross.

per plaque-forming unit (TAKAHASHI 1961; COPELAND, unpublished observations) of 10^{-6} for prototrophic markers, the probability of independent cotransduction of each marker would be about 10^{-12} . Also, the probability of isolating a transductant and a revertant together would be about the same or less. Compared to these estimates, the observed cotransduction frequency of 2×10^{-3} for *recA* and *aroA* take on significant proportions. Furthermore, the constitution of the recombinants selected in these crosses makes it unlikely that they are artifacts. In each case the association found was between a selectable positive marker (*Aro*⁺ or *Rec*⁺) and an unselected negative marker (*Rec*⁻ and *Aro*⁻ respectively). When other uninvolved markers were present, they remained unchanged as in the BC71 recombinant (*Rec*⁺, *Aro*⁻, *Trp*⁻ and *His*⁻). These linkage relationships, summarized in Figure 10, establish the location of these markers in agreement with other published reports (DUBNAU 1970; YOUNG and WILSON 1972), and indicate that linkage between *recA* and *aroA* is very weak.

A density transfer experiment was done to establish whether the *aroA* marker would replicate ahead of *recA*, since it is closely linked to *argA* but marginally linked to *recA*. The DNA samples for these gradients were obtained from cells of BC29, a 168 strain, following tryptophan starvation. During tryptophan starvation replication stops predominantly at the terminus of the chromosome. Subsequent replication is synchronized and ordered like that during spore germination and outgrowth or during growth of stationary phase cells (COPELAND, unpublished results). The first gradient (Figure 11) was sampled after net DNA synthesized was 17%. The reference marker *purA* has replicated but neither *recA* nor *aroA* shows significant replication activity. The next gradient was sampled after net DNA synthesized was 58% (Figure 12). The reference *purA* marker continued to replicate, but note that the *aroA* marker has replicated well ahead of *recA*, a marker located proximal to it on the current genetic map. These

FIGURE 10.—Linkage map showing the results of the two-factor transductional analysis using phage PBS1. The direction of the arrows indicates the selected to unselected markers analyzed. The broken lines indicate the weak linkage between *recA* and *aroA*.

FIGURE 11.—Transformation assay of a gradient taken when net DNA synthesized was 17% in BC29 cells. The cells were previously starved of tryptophan for 120 min in an H_2O medium before transfer to the D_2O medium. LL indicates peak of light fraction and HL that of the hybrid fraction.

FIGURE 12. Transformation assay of a gradient taken when net DNA synthesized was 58% in BC29 cells. Conditions as in Figure 11.

results eliminate mismapping of markers as being the cause for this discrepancy between their order of replication and their map position for the *ura-argA* region. They document the tenuous linkage relationship between *recA* and *aroA*, and they show that the site where replication disorder began is before *aroA* but after *recA*.

DISCUSSION

In this report, attention has been concentrated on documenting the discrepancies between replication order and map position. These are not wholly original findings, since they were first observed by O'SULLIVAN and SUEOKA (1967), although they interpreted their results as being caused by increasing asynchrony in distribution of replication forks. This interpretation cannot account for a distal marker replicating before a more proximal one according to the current model for the *B. subtilis* genome. They observed discrepancies between replication order and map position in the same regions documented in this report. For example, from among the genetic markers used in this study, they found that the *hisA* marker replicated before *purB*, its proximal neighbor on the genetic map. Also, they found that the *leu* marker replicated ahead of its proximal neighbor *ura*.

The results presented here further document these discrepancies in replication order and map position in these two regions of the current genetic map of *B. subtilis* (Figure 1), *thr-hisA* and *aroA-nia*. The earliest replicating marker to show this discrepancy was *thr* when compared to *purB* and *aroA* when compared to *recA*. Replication order was confirmed using both density transfer experiments and marker frequency analysis; thus, the replication order found is not an artifact of density labeling. The discrepancy between replication order and map position occurs in different cell lines of two commonly used strains of *B. subtilis*, 168 and W23, showing that it is not a function of a particular mutation or strain. Also, this discrepancy occurs in cells from a number of physiological states. It occurs following leucine starvation, when chromosomes are unaligned, and following tryptophan starvation, when chromosomes are aligned. The discrepancy between replication order and map position occurs when stationary phase cells of W23 are regrown and during germination and outgrowth of spores. At least in the case of *ura-argA* region, this discrepancy is not an artifact of mismapping. All of this information leads to the inescapable conclusion that disorder in replication relative to map position is a real occurrence which happens whenever the genome of *B. subtilis* is replicated.

A detailed examination of the linkage relationships in the *ura-argA* region was undertaken, since this region has been reported to be a continuously linked segment of the *B. subtilis* genetic map (DUBNAU 1970; YOUNG and WILSON 1972). The results are consistent with that interpretation. The only reasonable explanation for the replication order and map position discrepancy in this region is that an origin for new starts at replication exists between *recA* and *aroA*. The replication order results indicate that starts at replication can be made at this second origin before the replication fork from the first origin reaches that site. This interpretation would account for the apparent discrepancy in replication

order and map position observed in the density transfer experiments. It also would account for the higher marker frequency ratio for the apparently more distal marker compared to a proximal one, since the concept of proximal and distal are relative to the first origin near *purA*.

Demonstration of the replication order to map discrepancy in the *thr-hisA* region is variable. The closeness of this region to the origin near *purA* may complicate these determinations. The finding of bidirectional replication (GYURASITS and WAKE 1973; HARA and YOSHIKAWA 1973) in *B. subtilis* may explain this discrepancy between map position and replication order. The earliest marker found to replicate out of map positional order is *thr* when compared to *purB*. It is important to note that *purB* has not been genetically linked to other markers that replicate before or after it (DUBNAU *et al.* 1967; DUBNAU 1970; YOUNG and WILSON 1972). The *purB* position on the map is based solely on its replication order. This position may be in error, and its replication order may be a function of bidirectional replication in this region.

A mapping study using several heretofore unreported markers (J-A-LEPESANT, personal communication) has lead to a different interpretation of these results. The discrepancies between replication order and map position can be resolved by a major reorganization of the current genetic map into two arms joined to a common origin from which replication is bidirectional to a common terminus, which completes circularity. According to these results the *purB* segment and the *thr-cysB-hisA* segment are on opposite arms of this bidirectionally replicating chromosome. Also, the *ura-fur-recA* segment is located on one arm and the *aroA-argA-leu-8* segment on the other.

Our findings and those of others (GYURASITS and WAKE 1973; HARA and YOSHIKAWA 1973, and J-A LEPESANT, personal communication) necessitate a reconsideration of the current model for the organization of the *B. subtilis* genome. A consideration of different models to account for these results will be presented in another report (COPELAND, in preparation).

In conclusion, discrepancies between replication order and map position were found to be real. The results indicate that an origin for replication exists between *recA* and *aroA*. Replication from this second origin begins before the replication fork from the origin near *purA* reaches the *ura-recA* markers. The direction of replication from this second origin is the same as that from the origin near *purA*. The exact relationship between these two origins remains to be determined, but it appears that the origin near *purA* dominates over this second origin. New rounds of replication following aligned states such as during spore germination, regrowth of stationary-phase cells, and following tryptophan starvation begin from the origin near *purA*. Only after replication from this first origin is advanced, does replication start from the second origin near *aroA*.

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LITERATURE CITED

- CAIRNS, J., 1963 The bacterial chromosome and its manner of replication as seen by autoradiography. *J. Mol. Biol.* **6**: 208-213.
- COPELAND, J. C., 1969 Regulation of chromosome replication in *Bacillus subtilis*: Effects of amino acid starvation in strain 168. *J. Bacteriol.* **99**: 730-736. —, 1971a Regulation of chromosome replication in *Bacillus subtilis*: Effects of amino acid starvation in strain W23. *J. Bacteriol.* **105**: 595-603. —, 1971b Regulation of chromosome replication in *Bacillus subtilis*: Marker frequency analysis after amino acid starvation. *Science* **172**: 159-161.
- COPELAND, J. C. and J. MARMUR, 1968 Identification of conserved genetic functions in *Bacillus* by use of temperature-sensitive mutants. *Bacteriol. Rev.* **32**: 302-312.
- DENNIS, E. S. and R. G. WAKE, 1968 The *Bacillus subtilis* genome—studies on its size and structure. pp. 67-70. In *Replication and Recombination of Genetic Material*. Edited by W. J. PEACOCK and R. D. BROCK. Aust. Acad. Sci., Canberra.
- DUBNAU, D., 1970 Linkage map of *Bacillus subtilis*. pp. 39-45. In: *Handbook of Biochemistry*. Second edition. Edited by H. A. SOBER and R. A. HARTE. Chemical Rubber Co., Cleveland, Ohio.
- DUBNAU, D., C. GOLDTHWAITE, I. SMITH and J. MARMUR, 1967 Genetic mapping in *Bacillus subtilis*. *J. Mol. Biol.* **27**: 163-185.
- ERICKSON, R. J. and J. C. COPELAND., 1972 Structure and replication of chromosomes in competent cells of *Bacillus subtilis*. *J. Bacteriol.* **109**: 1075-1084.
- GYURASITS, E. B. and R. G. WAKE, 1973 Bidirectional chromosome replication in *Bacillus subtilis*. *J. Mol. Biol.* **73**: 55-63.
- HARA, H. and H. YOSHIKAWA, 1973 Asymetric bidirectional replication of *Bacillus subtilis* chromosome. *Nature New Biol.* **244**: 200-203.
- OISHI, M., H. YOSHIKAWA and N. SUEOKA, 1964 Synchronous and dichotomous replication of the *Bacillus subtilis* chromosome during spore germination. *Nature* **204**: 1069-1073.
- O'SULLIVAN, A. and N. SUEOKA, 1967 Sequential replication of the *Bacillus subtilis* chromosome. IV. Genetic mapping by density transfer experiment, *J. Mol. Biol.* **27**: 349-368.
- RAMAREDDY, G. V. and H. REITER, 1970 A new method for mapping the chromosome of *Bacillus subtilis* 168; evidence for the circularity of its chromosome. *J. Mol. Biol.* **50**: 525-532.
- SPIZIZEN, J., 1958 Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proc. Natl. Acad. Sci. U.S.* **44**: 1072-1078.
- SUEOKA, N. and W. G. QUINN, 1968 Membrane attachment of the chromosome replication origin in *Bacillus subtilis*. *Cold Spring Harbor Symp. Quant. Biol.* **33**: 695-705.
- TAKAHASHI, I., 1961 Genetic transduction in *Bacillus subtilis*. *Biochem. Biophys. Res. Comm.* **5**: 171-175. —, 1963 Transducing phages for *Bacillus subtilis*. *J. Gen. Microbiol.* **31**: 211-217. —, 1968. The isolation of nucleic acids from bacterial spores. pp. 99-100. In: *Methods in Enzymology*. Vol. XII, pt. B. Academic Press, New York.
- WAKE, R. G., 1963 Sequential replication of DNA in synchronously germinating *Bacillus subtilis* spores. *Biochem. Biophys. Res. Comm.* **13**: 67-70.
- YAMAGUCHI, K. and H. YOSHIKAWA, 1973 Topography of chromosome membrane junction in *Bacillus subtilis*. *Nature New Biol.* **244**: 204-206.

- YOSHIKAWA, H., 1970 Initiation of DNA replication in *Bacillus subtilis* II. Linkage between termini of parental strands and origins of daughter strands. *J. Mol. Biol.* **47**: 403-417.
- YOSHIKAWA, H. and N. SUEOKA, 1963a Sequential replication of *Bacillus subtilis* chromosome. I. Comparison of marker frequencies in exponential and stationary growth phases. *Proc. Natl. Acad. Sci. U.S.* **49**: 559-566. —, 1963b Sequential replication of *Bacillus subtilis* chromosome. II. Isotopic transfer experiments. *Proc. Natl. Acad. Sci. U.S.* **49**: 806-813.
- YOUNG, F. E. and G. A. WILSON, 1972 Genetics of *Bacillus subtilis* and other gram-positive sporulating bacilli. pp. 77-106. In: *Spores V*. Edited by H. O. HALVORSON, R. HANSON and L. L. CAMPBELL. American Society for Microbiology, Washington, D. C.

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