

GENETIC TRANSFORMATION AS A TOOL IN THE STUDIES OF DNA REPLICATION AND RECOMBINATION

(*transformation, replication, recombination*)

NOBORU SUEOKA

Department of Molecular, Cellular and Developmental Biology
University of Colorado
Boulder, Colorado 80302

SUMMARY

Mechanism of chromosome replication by the use of transformation and genetic recombination are reviewed in Bacillus subtilis. The order of gene replication can be determined by marker frequency analysis during exponential growth when the cells are asynchronous. Alternatively, in synchronized cells density transfer combined with transformation reveals the order of replication of markers. The frequency of the replicated genes is influenced also by their position relative to the origin of replication when the nutritional environment varies. A position effect may be shown by the sequential appearance of certain metabolic functions as a consequence of gene order on the chromosome. The origin and terminus of replication are membrane bound as seen by radioactive labeling and marker frequency. The membrane-DNA complex can be isolated and used for the study of the basic mechanics of replication. In vitro studies are feasible to compare initiation, elongation, repair and localization data with those obtained in vivo systems. In vitro analysis of recombination seems desirable but remains unsuccessful. An understanding of the basic biology of these functions promises important practical applications.

INTRODUCTION

Genetic transformation, first discovered in *Diplococcus pneumoniae* by AVERY, MACLEOD and McCARTY (1944), not only gave direct evidence that DNA is the chemical entity of genetic material, but provided a powerful new tool for studies on chromosome structure and replication and for mutation and recombination. The advantage of using transformation comes from its directness in analyzing the genetic constitution of DNA. The genetic marker indicates chromosomal location more precisely than any other means available including radioisotope labeling and electron mi-

microscopic observation. Over the years, we have worked on various aspects of chromosome replication in *Bacillus subtilis* using genetic transformation as a critical tool. In this article, I will present several studies on DNA replication and genetic mapping in which genetic transformation has provided salient information. The principles and techniques developed in this system should be applicable to many different systems in which genetic transformation is possible.

GENETIC REPLICATION ORDER

Using genetic transformation, over-all gene replication order can be determined by two different approaches.

(A) MARKER FREQUENCY ANALYSIS

This method (YOSHIKAWA and SUEOKA 1963a, SUEOKA and YOSHIKAWA 1965) is based on the following assumptions: 1) the temporal replication order is fixed; 2) the rate of replication at the fork is constant or nearly so; and 3) the population of cells is in nonsynchronous steady state growth (exponential growth).

As shown diagrammatically in Figure 1, markers close to the origin are more frequent than those close to the terminus. In principle, if we measure by transformation the relative marker frequencies in a DNA sample prepared from an exponentially growing population, we should be able to order genetic markers in their replication sequence. In practice, however, absolute transformation efficiency varies from marker to marker. This difficulty can be circumvented by using standard DNA samples in which all markers are equally frequent. DNA samples prepared from stationary cells of strain 23 (YOSHIKAWA and SUEOKA 1963a) and from spores of both strains 23 and 168 induced in potato extract medium (YOSHIKAWA, O'SULLIVAN and SUEOKA 1964) have been shown to satisfy these criteria.

The result indicated that the chromosome of *B. subtilis* has a replication origin close to the *ade6* marker with other markers replicated in a fixed order. The first over-all genetic map of *B. subtilis* was constructed using marker frequency analysis (YOSHIKAWA and SUEOKA 1963a). In addition, this method was crucial to the discovery of dichotomous replication; in rapidly growing cells (generation time 20') the second round of replication is initiated before the replication fork reaches the terminus (Fig. 2, YOSHIKAWA, O'SULLIVAN and SUEOKA 1964). In this case, the ratio between a marker close to the origin (*ade6*, now known as *purB6*) and the one close to the terminus (*metB5*) was 4 rather than the ratio of 2 seen with cells having a slower growth rate (generation time 40'). In later experiments, *ade16* (now known as *purA16*) which is located even closer to the origin (O'SULLIVAN and SUEOKA 1967) was used to reaffirm the initial observation. Dichotomous replication first provided evidence that bacteria can shorten the time required for chromosome replication and, in consequence, the cell generation time. This

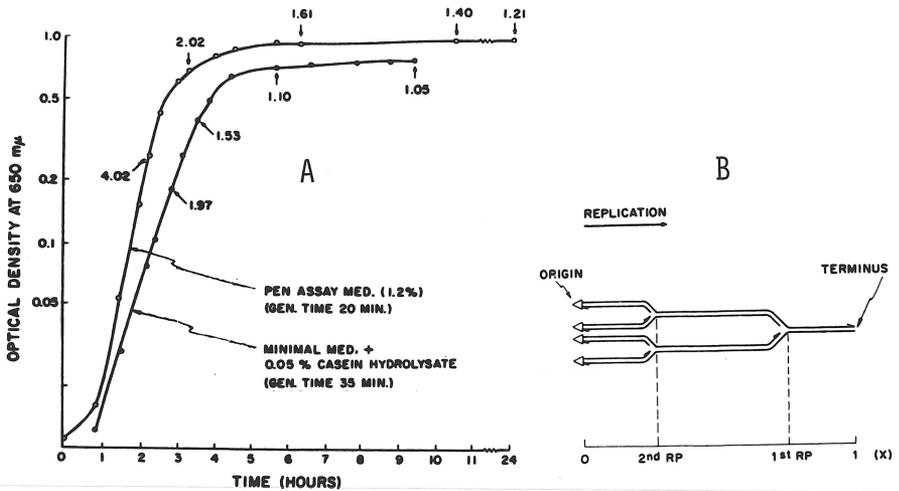


FIGURE 2. Dichotomous replication in *B. subtilis* (from YOSHIKAWA, O'SULLIVAN and SUEOKA 1964).

A. Growth rate and marker ratio. Growth rate and *purB6/metB5* ratio of strain W23 in standard medium (generation time, 40') and a Penassay medium (generation time, 20') are compared. The *purB6* marker is located close to the origin and *metB5* to the terminus. The ratios were obtained by normalizing against a standard DNA prepared from stationary phase strain W23. Figures on the growth curve represent the normalized *purB6/metB5* ratios.

B. Schematic representation of dichotomous replication of the chromosome. Before the first replication position (RP) reaches the terminus, the second RP starts from the origin. In the simplest steady state model for an exponentially growing population, the distance between the first and second RP is half of the chromosome and each replication point proceeds at a constant rate. In reality, the two replication points at the second RP may not necessarily be at exactly the same position. This model for dichotomous replication was later validated by experiments combining synchrodensity transfer and transformation (OISHI et al. 1964, QUINN and SUEOKA 1970).

of marker frequency. Defining two parameters (X = the position of a marker on unitary scale of chromosome, Fig. 1; n = average number of replication positions, Figs. 2 and 3), the frequency of marker X , $g_n(X)$, can generally be calculated as:

$$g_n(X) = 2^{n(1-X)} \quad (\text{SUEOKA and YOSHIKAWA 1964})$$

Thus, relative frequency between two markers, X1 and X2, is:

$$g_n(X1)/g_n(X2) = 2^{n(X2-X1)}$$

Experimentally, $n = 1$ for a 40 minute cell generation time and $n = 2$ for a 20 minute generation time. Marker frequency analysis can be applied to the exponentially growing steady state condition in which artificial culture conditions have not been imposed on the cells. Since the technique depends on replication order, the result cannot by itself differentiate between several configurational alternatives for replication (e.g., unidirectional vs. bidirectional, or single-replicon vs. multi-replicons) as was originally pointed out by YOSHIKAWA and SUEOKA (1963a). Transformation is not the only way to measure marker frequency. BIRD et al. (1972) used a prophage Mu-1 which occupies various sites on the host chromosome and a prophage λ with a fixed site. By comparing relative frequency of these two prophage DNAs as a function of the position of prophage Mu-1 on the *E. coli* chromosome, they established the origin of replication and the replication order of the *E. coli* markers. The work gave the first critical evidence for bidirectional replication in bacteria.

The following observations should aid in the planning and execution of marker frequency analysis:

(1) Marker ratios are not DNA concentration dependent (YOSHIKAWA and SUEOKA 1963a).

This theoretically reasonable and also fortunate experimental fact makes marker ratio analysis both powerful and simple. Frequently, some samples have DNA concentrations above the linear dosage response of transformation (usually above 0.1 $\mu\text{g/ml}$). This situation, however, will not affect the analysis, as long as interpretations are based on ratios of different markers. Frequently encountered cases are those of transformation profiles of DNA fractionated by CsCl density gradient centrifugation, sucrose gradient centrifugation, zone electrophoresis and various column chromatographies in which transformation in peak areas is often performed with DNA concentrations above the linear response range. An interesting example was recently seen in the case where membrane bound DNA and free DNA were separated by CsCl-sucrose double gradient centrifugation and both fractions were analyzed by transformation (SUEOKA and HAMMERS 1974). In this case, the free DNA fraction contained lysozyme which had been used to lyse the cells. The transformation efficiency of the free DNA fraction was, therefore, considerably lower than that of the membrane DNA fraction. This difference in efficiency was eliminated by deproteinizing each fraction with phenol. There was, however, no significant difference in marker ratios before or after phenol treatment.

(2) Relatively large statistical errors are intrinsic to the ratio analyses.

This is particularly true for the double ratio analysis. Statistical error estimations for single and double ratio analyses have been formulated (SUEOKA and YOSHIKAWA 1964). In order to obtain meaningful results, experiments should be care-

fully designed in duplicate.

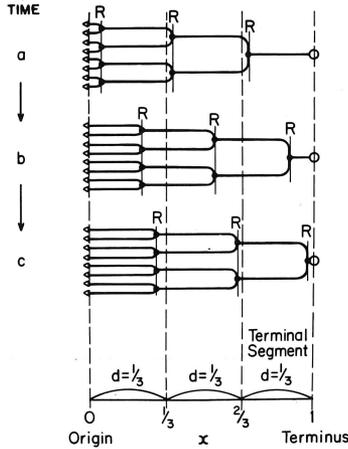


FIGURE 3. Dichotomous (symmetric) initiation of the chromosome (from SUEOKA 1971). Schematic representation of dichotomous replication with three replication positions per chromosome ($n=3$) is shown as an example. Each segment has a length of $d = \frac{1}{n}$, thus $1/3$ in the diagram. This distance d corresponds to the initiation interval. Note that each chromosome has three replication positions (R) and seven replication points (O); in general $2^n - 1$. The terminal segment occupies between $2/3$ and 1 ; in general the terminal segment is the segment of the chromosome between $1 - \frac{1}{n}$ and 1 in which $n > 0$ and not limited to integral numbers. The mode of connection between the origins and the terminus is not defined. R: replication position; \bullet : replication point; \circ : terminus; \triangleleft : origin. Functions for age distribution, $f(x)$, and for the marker frequency, $G(X)$, in the general form are:

$$f(x) = n(\ln 2) 2^{n(1-x)}$$

$$G(X) = 2^{n(1-X)}$$

where n represents the number of replication positions per chromosome, e.g., in both *B. subtilis* and *E. coli*, $n = 1$ for 40' cell generation time and $n = 2$ for 20'.

(B) SYNCHRODENSITY TRANSFER ANALYSIS

Replication order can also be analyzed by combining synchronization of chromosome replication and a Meselson-Stahl type density transfer experiment. In the latter, the cells are transferred from isotopically or otherwise heavy (or light) me-

dium to light (or heavy) medium, which in turn changes the density of DNA drastically. The density change can be detected by CsCl density gradient centrifugation. Replication can be followed through marker transfer from parental DNA to daughter DNA and then to doubly replicated DNA by genetic transformation. Marker replication order was followed in *B. subtilis* during re-growth from stationary phase (YOSHIKAWA and SUEOKA 1963b), during spore germination (OISHI et al. 1964, O'SULLIVAN and SUEOKA 1967) and during reinitiation of mutants temperature sensitive for initiation (O'SULLIVAN and SUEOKA 1972, WHITE and SUEOKA 1973). The results confirmed the replication order which had been obtained by the marker frequency analysis.

A rigorous criterion for the synchrony of chromosome replication is to recognize the replicated regions of the chromosome. For this purpose, density transfer and transformation are two essential techniques. This synchronicity transfer technique provides a particularly precise replication order for those markers close to the origin. The ordering of markers close to the terminus, however, is less feasible because of synchrony breakdown. The method does not give information on the mode of chromosome replication in undisturbed exponentially growing cultures, where all markers should transfer to the replicated DNA at an equal rate (SUEOKA and YOSHIKAWA 1965, WHITE and SUEOKA 1973).

MARKER FREQUENCY AND POSITION EFFECT

Adaptability to the nutritional environment confers a most important selective advantage on bacteria. The cell which responds quickly and efficiently, for example, to a sudden increase in nutrients can propagate and become the dominant cell type in the population. The ability to adapt to the opposite situation, i.e., a poorer environment, without unbalancing nuclear and cellular division may be no less important. A selective advantage may result when genes whose product concentrations are directly proportional to the cell growth rate are located near the chromosome origin, since those loci nearer the origin would be more frequent for any given time (SUEOKA 1969). Structural genes for ribosomes and tRNA are potential candidates for this type of position effect. The finding that most of the structural genes of ribosomal RNAs, ribosomal proteins, and tRNAs are located in the vicinity of the replication origin in *B. subtilis* (OISHI and SUEOKA 1965, DUBNAU et al. 1965, OISHI et al. 1966, SMITH, et al. 1968, HARTFORD and SUEOKA 1970, CHOW and DAVIDSON 1973) and *E. coli* (YU et al. 1970, BIRNBAUM and KAPLAN 1971, MATSUBARA et al. 1972) substantiated this hypothesis. Needless to say, this hypothesis does not preclude the existence of the on-and-off type of control of genes involved in protein synthesis (e.g., ribosomal genes) as seen in relaxed and stringent control of rRNA synthesis (see review article by HASELKORN and ROTHMAN-DENES 1973). When the transcription of these genes is switched on, the amount of product should reflect gene dosage.

GENE ORDER AND FUNCTION

Another possible gene position effect occurs where gene order on the chromosome may regulate gene function. A possible illustration of this type of position effect may be found during spore germination in *B. subtilis*. Bacterial spores are metabolically inert and during outgrowth a series of metabolic events occur in a certain order (see review article by HALVERSON, VARY and STEINBERG 1966). As mentioned above, in *B. subtilis* spores prepared in potato extract medium, the chromosome is in completed form and, during outgrowth, replicates from the origin. Syntheses of RNA, protein, and DNA during synchronized *B. subtilis* spore germination show a sequential pattern (Fig. 4, ARMSTRONG and SUEOKA 1968). RNA synthesis begins first and shows two different phases. Ribosomal and soluble RNA synthesis starts in the first phase, followed by messenger RNA in the second phase. Leucine incorporation into protein begins in the second period and DNA synthesis later (Fig. 5).

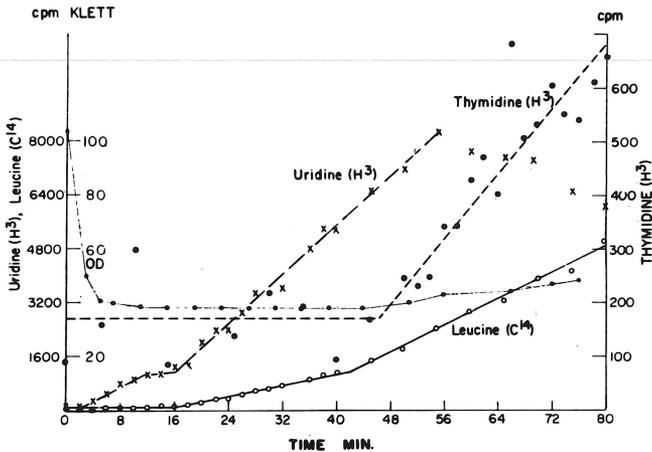


FIGURE 4. Kinetics of uridine, leucine, and thymidine incorporation during spore germination (from ARMSTRONG and SUEOKA 1968). At zero time spores of strain 23 *thy⁻his⁻* were added to 5 ml of germination medium containing radioactive leucine (0.4 $\mu\text{C}/\text{ml}$) and uridine (0.4 $\mu\text{C}/\text{ml}$) or thymidine (2 $\mu\text{C}/\text{ml}$), and 0.1-ml samples were taken at the times indicated. This figure is the composite of two separate experiments and shows the reproducibility of germination since OD and leucine incorporation matched very well. The germination medium consists of Spizizen salts (ANAGNOSTPOULOS and SPIZIZEN 1961), L-alanine (100 $\mu\text{g}/\text{ml}$), L-histidine (100 $\mu\text{g}/\text{ml}$), thymine (50 $\mu\text{g}/\text{ml}$), casamino acids (500 $\mu\text{g}/\text{ml}$), yeast extract (200 $\mu\text{g}/\text{ml}$) and 0.5% glucose. Germination was carried out at 37°C with shaking. OD was monitored by a Klett-Summerson colorimeter. Essentially similar pattern was obtained with a defined medium, GM11 (KENNETT and SUEOKA 1971, ARMSTRONG, KENNETT and SUEOKA 1969).

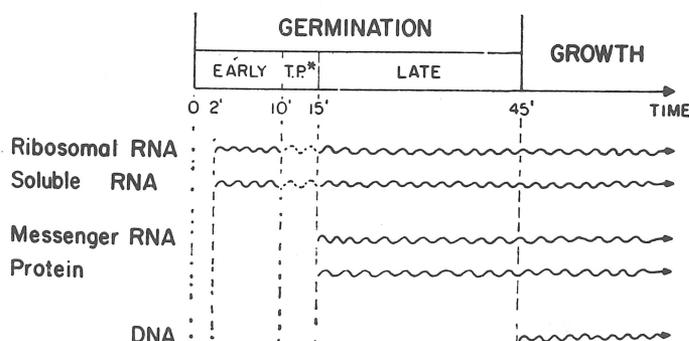


FIGURE 5. Schematic representation of events during *B. subtilis* spore germination. Since it is unknown if soluble and ribosomal RNA synthesis occurs during the transition period, a dashed line is used to indicate this. Zero time represents the time of adding spores to a prewarmed germination medium. *Transition period.

We have examined the production of five enzymes (sucrase, trehalase, ornithine transcarbamylase, aspartate transcarbamylase and threonine dehydratase) during spore outgrowth in *B. subtilis* (Fig. 6, KENNETT and SUEOKA 1971). The enzyme activities appeared in a sequential manner. With the exception of ornithine transcarbamylase, the activities appear before DNA replication begins in the order corresponding to the replication order of their genetic loci. Ornithine transcarbamylase activity does not appear in the first round of increases in enzyme activity but does in the second round after DNA synthesis commences. Moreover, the appearance of this enzyme activity then occurs at a time that would be expected from its relative position on the genetic map (Fig. 6). It is possible that a high concentration of L-arginine is present during outgrowth due either to its high concentration in the spores (NELSON et al., 1969) or to peptide or protein hydrolysis (LEE and ORDAL 1963). Arginine thus may repress the synthesis of ornithine transcarbamylase in the first round.

CHROMOSOME ATTACHMENT TO MEMBRANE

An attachment of the bacterial chromosome to the cell membrane, as first proposed by JACOB, BRENNER and CUZIN (1963), suggests a number of interesting possibilities for chromosome organization and replication. As pointed out by these authors, it permits localization of the chromosome in the cell and provides a basis for mechanical separation of the two daughter chromosomes. It also provides a special metabolic environment for chromosome replication and initiation. Substantial evidence has now accumulated to support a membrane-chromosome association (see review by GOULIAN 1971).

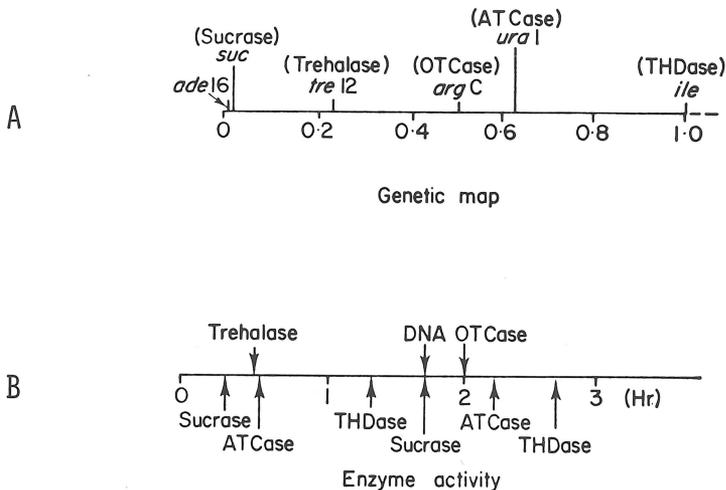


FIGURE 6. Relationship of genetic markers (O'SULLIVAN and SUEOKA 1967, DUBNAU et al. 1967) (A) to order of enzyme steps during spore outgrowth (B) (from KENNETT and SUEOKA, 1971). The beginning of each increase in activity is indicated in B. The time of initiation of chromosome replication is also indicated. The *suc* marker is now designated as *sacA24* (LEPESANT et al. 1972). For positions of *tre12*, *argC*, *ura1* on the current circular map, see HARFORD (1975). There have been no changes in replication order of these markers.

The first experimental indication of membrane attachment of the replication origin and terminus was obtained in *B. subtilis* using genetic transformation; origin attachment to the membrane was substantiated by radioactive labeling experiments (SUEOKA and QUINN 1968). It was shown that the membrane-associated DNA fraction was enriched for markers close to the origin (Fig. 7) and the radioactive label at and near the origin remained bound to the membrane. Origin and terminus attachment to the membrane has since been confirmed both in *B. subtilis* and *E. coli* (SNYDER and YOUNG 1969, O'SULLIVAN and SUEOKA 1972, SUEOKA and HAMMERS 1974).

Isolation and characterization of the DNA-membrane complex are in the preliminary stages of development. Separation of the complex from the bulk of the membrane and DNA was only recently begun. The use of the CsCl-sucrose double gradient permits separation of the DNA-membrane complex from the major portion (>95%) of the membrane (Fig. 8, SUEOKA and HAMMERS 1974). An important experiment in the characterization of the complex is the transformation assay which identifies the regions of the chromosome which form a stable association with the membrane. The DNA in the complex obtained by CsCl-sucrose gradient is enriched for markers close to the origin and the terminus; all other mark-

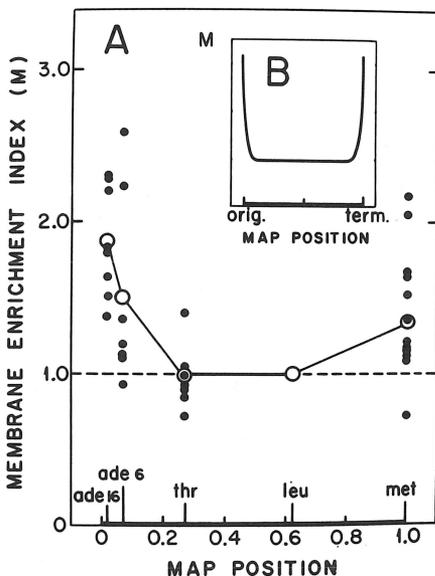


FIGURE 7. Enrichment of genetic markers in the membrane fraction of *B. subtilis* (from SUEOKA and QUINN 1968).

(A) The membrane-enrichment index (M) is defined as $M = (X_m/X_f)/(S_m/S_f) = (X_m/S_m)/(X_f/S_f)$, where X_m and X_f are transformants of marker X in membrane-bound and membrane-free DNA of marker S (standard, leucine marker in the present analysis) in membrane and free fractions, respectively. The large open circle is the average of values for a marker. It is noted that variability of the index for the leucine marker is necessarily added to variability of the other markers.

(B) Expected distribution of M , assuming the attachment of the origin and the terminus to the membrane.

The medium used (GM11) is a synthetic medium originally developed for spore germination (KENNETT and SUEOKA 1971). Cell generation time at exponential phase is 60 min.

Similar data have been accumulated for more genetic markers and for different generation times, the results of which are consistent with the above conclusion (SUEOKA et al. 1973).

ers, however, are found in this complex. Thus the DNA-membrane complex fraction seems to include at least the initiation, replication and termination complexes. Analysis by SDS-polyacrylamide gel electrophoresis shows the protein species in this fraction to be similar to the major membrane fraction proteins except for several proteins which characteristically are enriched in the DNA-membrane complex (IMADA and SUEOKA 1975).

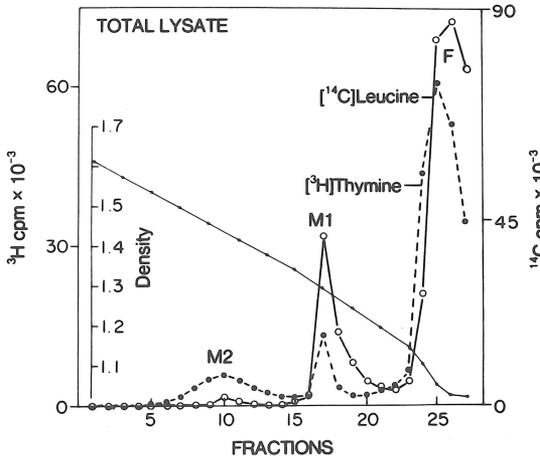


FIGURE 8. CsCl-sucrose double gradient centrifugation of a sheared cell lysate ($[^3\text{H}]\text{DNA}$, $[^{14}\text{C}]\text{protein}$) (from SUEOKA and HAMMERS 1975). *B. subtilis* 168TT was labeled by growth in the presence of $[^3\text{H}]\text{thymine}$ (10 $\mu\text{Ci/ml}$, specific activity 49.5 Ci/mmol), thymine (2 $\mu\text{g/ml}$), and $[^{14}\text{C}]\text{leucine}$ (1 $\mu\text{Ci/ml}$, specific activity 304 mCi/mmol, and a sheared lysate was prepared as described in Materials and Methods of SUEOKA and HAMMERS (1975). A 2-ml portion of the lysate was layered on top of a 15-ml CsCl-sucrose double gradient and the tube was centrifuged for 30 min in a 17-ml SW 27 rotor (4° , 25,000 rpm Spinco model L2 ultracentrifuge). Twenty-seven fractions (0.6 ml per fraction) were collected from the bottom of the tube. Aliquots of 0.1 ml were analyzed for radioactivity and one drop from each fraction was used to measure refractive index (n_D^{20}) with an Abbey refractometer. The remainder of each sample was frozen. The density of each fraction was calculated from the refractive index.

Further work in this area includes structural studies of the replication origin of the chromosome and the structural elements of initiation-, replication-, and the termination-complexes, all of which are likely to be embedded in the membrane. Our current model of chromosome attachment to the cell envelope in *B. subtilis* is shown in Figure 9 (IMADA et al. 1975). Genetic transformation will undoubtedly remain extremely useful and critical for these studies.

IN VITRO CHROMOSOMAL SYNTHESIS

Chromosome replication in bacteria is the result of an organized interplay of enzymes and structural components and is, of necessity, quite complex. The results of *in vitro*, therefore, are liable to misinterpretations due to various artifacts. Genetic transformation in *B. subtilis* provides a unique opportunity

for *in vitro* replication study through direct analysis of the product in terms of its chromosomal location as well as its biological (transforming) activity, and to compare it with that of *in vivo* synthesis, thus providing a system much less liable to artifactual interpretation.

Taking advantage of this situation, we (MATSUSHIKTA et al. 1971) have extended, using a toluenized *B. subtilis* cell system, the original observations of MOSES and RICHARDSON (1970) in *E. coli* as follows:

(1) The DNA replicated in the toluenized system which amounts to 10% of the total genome has full biological (transforming) activity.

(2) Replication is the result of DNA elongation at the replication forks which existed in the cells prior to toluenization.

(3) Initiation at the chromosomal origin does not occur in the toluenized system.

We have formulated experimental conditions for *in vitro* initiation, elongation (SUEOKA et al. 1973) and repair (MATSUSHITA and SUEOKA 1974), using the transformation system. Toluenized cells can apparently elongate and repair DNA, but *do not* initiate chromosome replication.

a) INITIATION TEST

Exponentially growing, thymine-requiring cells uniformly labeled with ^{14}C -thymine are toluene treated and *in vitro* DNA synthesis is performed with dBUTP in place of TTP. If the origin marker (e.g., *purA16*) is found in hybrid DNA to the same extent as other markers, the system initiated *in vitro*. For the experimental principle and result in toluenized cells, see SUEOKA et al. (1973).

b) ELONGATION TEST

Exponentially growing thymine-requiring cells are washed and suspended in a thymine-less medium, kept for 30' and brought to an *in vitro* system with dBUTP replacing TTP. In this condition, all initiation processes apparently have been completed at most chromosome origins during thymine starvation. When these thymine starved cells are brought into an *in vitro* system and supplied with deoxyribonucleoside triphosphates (dBUTP instead of TTP) and ATP, elongation can begin at the origin and the replication fork should quickly reach the loci close to the origin. Pre-existing replication forks also restart replication, but in this case there will be no selective replication of particular markers. Therefore, if an origin marker (e.g., *purA16*) is found in the hybrid DNA in excess of markers from middle and late replicating regions (e.g., *thr5*, *leu8*, *metB5*), elongation must have occurred *in vitro* (SUEOKA et al. 1973).

The above two tests are free of repair synthesis compli-

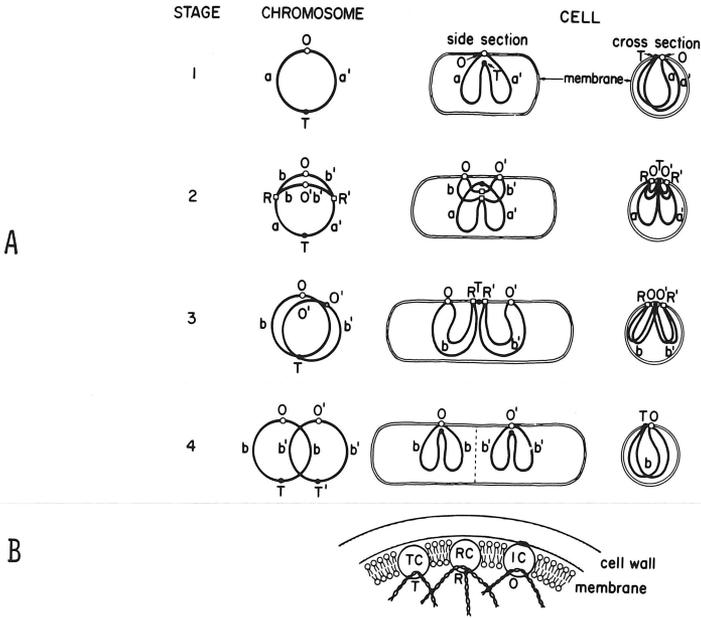


FIGURE 9. A model of chromosome attachment to the cell envelope. This model is defined as shown in the two sets of diagrams, A and B (from IMADA et al. 1975).

- (A) Replication stages of the chromosome and association of the chromosome with the membrane.
 O, O': Replication origins. R, R': Replication forks.
 T, T': Replication termini. a, a': Left and right halves of the nonreplicated chromosome. b, b': Left and right halves of the replicated part of the chromosome. The terminus is assumed, for the sake of convenience, to be located equidistant bidirectionally from the origin since in *B. subtilis* the location of the terminus is still unresolved (WAKE 1973, O'SULLIVAN et al. 1975, HARFORD 1975, LEPESANT-KEJZLAROVA et al. 1975).
- (B) Association of chromosome with the membrane at initiation, replication and termination complexes. Note that in this model the initiation complex (IC) is not only embedded in the membrane but is also attached to the cell wall. In contrast, the replication complex (RC) and the termination complex (TC) are not attached to the cell wall, permitting them greater mobility on the membrane.

cations since density transfer and transformation are both used. DNA synthesis can be measured by the amount of ^{14}C radioactivity in the hybrid DNA.

c) REPAIR TEST

As shown by PETTIJOHN and HANAWALT (1964), newly repaired DNA is dispersed in short stretches, and the use of ³H-BU does not substantially change the density of repaired DNA. ³H-radioactivity in the parental density region is then indicative of repair synthesis. DNA polymerase I dependent repair can be specifically tested for by the addition of p-chloromercuribenzoic acid (50 μM) to the reaction mixture (MATSUSHITA and SUEOKA 1974).

d) LOCALIZATION TEST

DNA replication and particularly repair synthesis should be distinguishable from soluble DNA synthesis, i.e., that occurring on fragmented chromosomes. The latter type of DNA synthesis will occur whenever DNA pieces having single stranded gaps or tails and 3' hydroxyl ends are available as the primer and the opposite strand is available as the template. This situation is a biologically meaningless artifact and control experiments should be done to avoid confusion. In the localization test, chromosomes or chromosome-carrying cells are separated from the soluble fraction by centrifugation or by filtration and the acid precipitable counts in the soluble fraction are measured. Applications of this test to toluenized *B. subtilis* system have been reported (MATSUSHITA and SUEOKA 1974).

IN VIVO AND IN VITRO RECOMBINATION

Studies on the mechanism of transformation (or recombination) until now have been carried out *in vivo*. The use of genetic markers and the detection of recombination by transformation have been the major techniques (HOTCHKISS and GABOR 1970, DUBNAU and CIRIGLIANO 1974). Hitherto unsolvable problems of recombination will become amenable to experimental analysis if an *in vitro* recombination system can be developed. Toluene treated recipient cells have been used as an *in vitro* system under various conditions (IMADA and SUEOKA 1974). Thus far, no recombinant molecules between two linked markers *hisB2* and *trp2* have been observed. The toluene treated cells can, however, accept donor DNA which remains double stranded and biologically active. The inability of toluenized cells to perform recombination may mean that the intact membrane is in some way involved in the processes of recombination.

CONCLUSION

Genetic transformation in *Bacillus subtilis* first developed by SPIZIZEN (1958) has provided a unique and powerful tool for analyzing various features of chromosome replication and recombination. A number of new principles such as marker frequency vs. marker position, new mapping methodology, marker frequency vs. growth rate, symmetric configuration of multifork chromosome, origin and terminus attachment to membrane, assessment of *in vitro* DNA replication, and recombination studies have emerged from

studies in which genetic transformation has played a critical role.

Promising future studies in which genetic transformation can play a critical role are: a) *in vitro* studies of DNA replication and recombination, b) the role of the bacterial membrane in DNA replication, initiation and termination, and c) structural analysis of replication origin of the chromosome. The knowledge obtained by these studies not only contributes to the progress of basic molecular biology but also to its application to the genetic engineering of industrially useful bacteria.

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Noboru Sueoka



N. Sueoka and E. Garber



N. Sueoka



Don Miles



B. Cumbie and D. Mertz



E. Chargaff and R. B. Helling



Sandy Bolen

R. C. King

J. D. Cassidy