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## *SEQUENCE OF ENZYME SYNTHESIS AND GENE REPLICATION DURING THE CELL CYCLE OF BACILLUS SUBTILIS**

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In a previous communication we reported that certain enzymes were synthesized only during parts of the cell cycle in *Escherichia coli* and *Bacillus subtillis*.[1] Periods of little net enzyme synthesis alternated with periods of rapid synthetic activity. The synthesis of each of the enzymes studied was taking place in a manner which we have termed *autogenous*,[2] that is, the enzyme synthesis occurred under the influence of the control mechanisms present in the normally growing cell (i.e., subject to the influence of feedback loops). In addition, we measured the *potential* for synthesis of certain other enzymes (i.e., the rate of synthesis under induced or derepressed conditions). This potential proved to vary discontinuously. It remained constant for the length of a cell cycle and then quite abruptly doubled.

Thus, in both situations there occurred a cyclic event which was clearly discernible. In the case of autogenous synthesis, this event was a period of synthetic activity during the normal growth cycle. When synthetic potential was measured, it was the periodic doubling of the cell's ability to make the enzyme.

Since it has been shown that the *B. subtilis* genome is replicated sequentially,[3] one can attempt to correlate the order of synthetic events with the order of replication of genes. For this purpose the autogenous synthesis of four enzymes of *B. subtilis* has been studied [histidase, aspartate transcarbamylase (ATCase), ornithine transcarbamylase (OTCase), and dehydroquinase (DHQase)]. In addition, the potential for sucrase synthesis was measured. All the information

available on the times of syntheses of the five enzymes was then used to order their syntheses in a linear sequence as a function of the cell division cycle. Mutants unable to produce these particular enzymes were obtained whenever possible and these were mapped genetically by the method of Yoshikawa and Sueoka.[3] The order of enzyme syntheses was shown to correspond well, but not perfectly, to the order of their respective genetic markers on the *B. subtilis* genome.

*Materials and Methods.—Strains:* *B. subtilis* W23, a wild-type strain, was used in all synchrony experiments to measure the autogenous bursts of enzyme activity. The following strains were used to map the various enzymes. Strain 168 *tryp*$^-$ *suc*$^-$ was isolated from a 168 *tryp*$^-$ strain obtained from Dr. L. Weed and the terminal *meth*$^-$ marker of Yoshikawa and Sueoka[3] introduced by transformation. Strain SB 121 (*tryp*$_2^-$ *shik*$^-$), which lacks DHQase, and strain SB 5 (*tryp*$_2^-$ *his*$^-$ *ura*$^-$), which lacks ATCase, were obtained from Dr. E. Nester. Strain 168-2 (C4) (*leu*$^-$ *tryp*$_2^-$ *arg*$^-$), which lacks OTCase, was obtained from Dr. J. Marmur. To measure relative changes in transforming activity during the cell cycle, 168 *tryp*$_2^-$ *suc*$^-$ *meth*$^-$ and 168 *tryp*$_2^-$ *his*$^-$ were used as recipients.

*Growth and synchronization:* Cultures were grown and synchronized as reported previously.[1]

*Enzyme assays:* Histidase, sucrase, and ATCase were measured as reported previously.[1] OTCase was measured by the method developed for ATCase,[4] substituting 10 $\mu$moles/ml of ornithine for aspartic acid. DHQase was assayed by the method of Mitsuhashi and Davis[5] using a crude culture filtrate of *E. coli* 170-27, a dehydroquinate excreter, as substrate source.

*Genetic mapping:* Genetic mapping was done by the method of Yoshikawa and Sueoka.[3] The exponential and stationary transforming DNA's from W23 were the gift of Dr. H. Yoshikawa.

*Transformation:* Transformation was done by a modification of the method of Spizizen.[6, 7] The partially purified DNA used to measure variation in relative transforming activity during the cell cycle was prepared as follows. Five-ml aliquots of synchronous culture were centrifuged and resuspended in 1 ml of 0.15 *M* NaCl containing 0.1 *M* EDTA, pH 8.0. They were heated 15 min at 60° to kill viable cells. Lysozyme was then added at a concentration of 1 mg/ml and the mixture incubated 30 min at 37°. Duponol was added to a final concentration of 2.5% and sodium perchlorate to 1 *M*. The samples were partially deproteinized by shaking 30 min at room temperature with an equal volume of $CHCl_3$: isoamyl alcohol which had been mixed in the proportion of 24:1. After centrifugation the aqueous phase was made 10% in NaCl and stored in the cold prior to use in transformation.

*Results.—Autogenous synthesis of enzymes:* The syntheses of ATCase, OTCase, histidase, and DHQase, in synchronous cultures growing on histidine, have been investigated. OTCase and DHQase were found to be synthesized in the stepwise fashion reported earlier for ATCase and histidase[1, 2] (Fig. 1). Autogenous synthesis appears to have the following characteristics: (*a*) Each enzyme increases in a stepwise fashion. They exhibit periods of rapid activity increase alternating with periods of no net synthesis. (*b*) The time required for a full cycle in enzyme synthesis is approximately equal to a cell generation time. (*c*) During each generation the total activity of each enzyme increases by a factor of approximately 2.0. (*d*) The relative time in the cell cycle at which each enzyme increases is similar in succeeding cycles but differs for each enzyme.

*Sequence of autogenous enzyme bursts:* An average order can be assigned to the steps which characterize the autogenous synthesis of each of the enzymes studied. This was done by comparing the syntheses of more than one enzyme during the growth of a synchronous culture, and correlating the information thus obtained from several such cultures. The time at which a step was half completed (i.e., the quantity of enzyme was half doubled) was determined for each step, and plotted in Figure 2. This enzyme half-doubling time (HDT) was chosen as a standard of

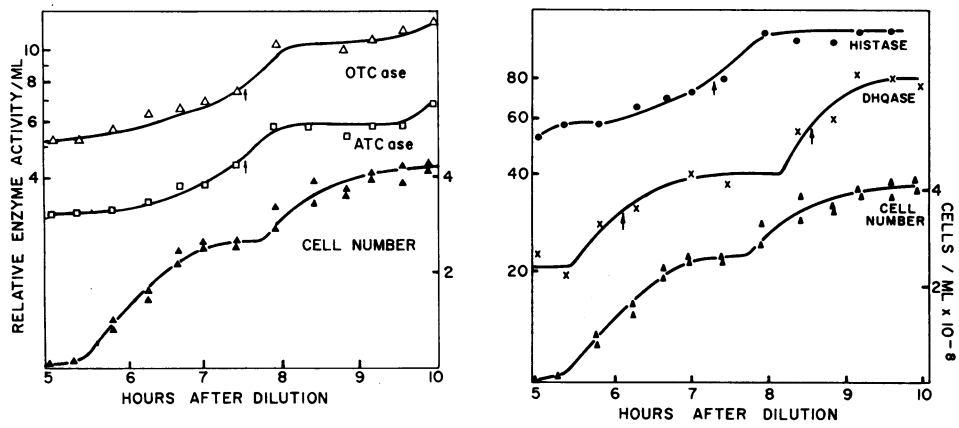


FIG. 1.—Autogenous synthesis of enzymes in synchronous *B. subtilis* cultures. Samples were taken at regular intervals, commencing 5 hr after the dilution of the stationary culture into fresh medium. Further protein synthesis in the samples was prevented by the addition of 50 μg/ml of chloramphenicol. At the same time, samples were taken for plate counts. The levels of OTCase and ATCase in each of the samples were determined in the first experiment (*a*), and those of histidase and DHQase in the second (*b*). The activity of each enzyme, expressed in relative units, is plotted against the time of sampling. The arrows mark the enzyme half-doubling times (HDT's).

comparison both to correct for heterogeneity in the population and because it was easier to estimate than the time at which synthesis was initiated.

The various HDT's were arranged in a linear array from 0 to 1. The origin was taken as the average HDT of the potential for sucrase synthesis, since the genetic map position of sucrase proved to be 0.0 (see below). All HDT's were converted to fractions of the length of the cell cycle in which they occurred, since these cycles varied in length from one to the other. The length of a cycle was estimated from the time between consecutive HDT's of histidase, an enzyme which was measured in all experiments. With the average HDT of sucrase equal to 0.0, the average HDT of histidase for each experiment was chosen as 0.54 (that is, 0.54 of the time through the cycle). The scatter in the single-cycle HDT's of histidase (Fig. 2) shows the variation possible among the two or three cycles of a single experiment. The HDT's of other enzymes obtained for each cycle were normalized to this average HDT for histidase of 0.54 for the experiment in which they were measured.

The results of five such experiments, totaling 11 cycles, are compiled in Figure 2. These include the experiments reported in Figure 1 as well as those reported in previous communications.[1, 2] It is apparent that the HDT's for each enzyme cluster in a particular segment of the cell cycle. The average HDT for each enzyme was calculated and these were compiled in a single linear array (Fig. 3), an enzyme-production map. It should be noted that this represents an average order and spacing. In any particular experiment, enzymes whose average positions are close together, e.g., ATCase and histidase, might be reversed in position, although remaining close together. Thus, the order of syntheses of enzymes, although roughly determined, is not absolutely fixed, as is the replication of the corresponding genes. Another uncertainty is that DHQase could equally well be placed at 0 or 1.

*Genetic mapping data:* Negative mutants for OTCase, ATCase, DHQase, and

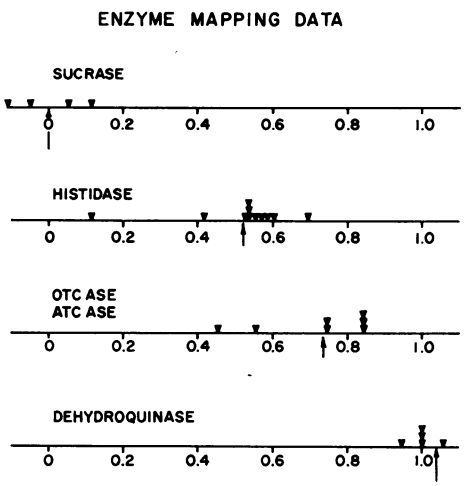


FIG. 2.—Enzyme production data. Each point represents the HDT of an enzyme in one cycle, taking the average HDT for histidase in that entire experiment equal to 0.54. The arrows indicate the mean HDT for each enzyme. OTCase and ATCase are plotted on the same line since their HDT's were indistinguishable in all experiments.

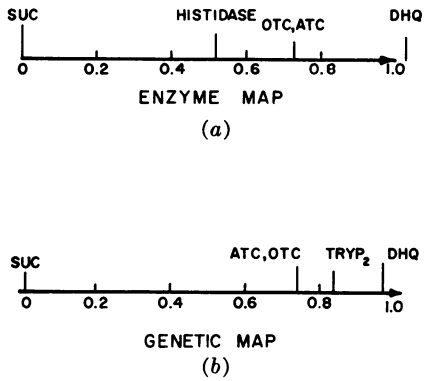


FIG. 3.—Enzyme-production and genetic maps. The points on the enzyme map are the average enzyme half-doubling times (see Fig. 2). The genetic map is compiled from data in Table 1.

sucrase were available. Each was mapped with respect to methionine, the most distal marker known for *B. subtilis* 23. The theory and limitations of this method of mapping have been considered by its originators.[8] Since the final values are calculated from a ratio of ratios, the sampling errors to which the procedure is liable are subject to considerable magnification. They calculate that two thousand colonies of each transformant class must be counted in order to assure a theoretical precision of $\sigma = 0.07$, i.e., 7 per cent of the genetic map length. In each experiment reported here, the standard deviation was 0.1 or less. Most experiments were done on double or triple mutants containing the $tryp_2$ locus as well as the unknown one. $Tryp_2$ was carefully mapped with respect to the terminal methionine marker (Table 1).

Although Oishi, Yoshikawa, and Sueoka have reported the map position of $tryp_2$ as 0.64, experiments conducted in our laboratory, including internally consistent three-point experiments, showed it to map at 0.85. In addition, a locus concerned with histidine biosynthesis, $his_1$, when mapped with respect to $tryp_2$ (0.85), mapped at 0.53, in agreement with a value previously reported by Yoshikawa and Sueoka.[3]

The results obtained from a number of mapping experiments are compiled in Table 1. The average value for each marker was calculated, and the data are presented as a linear map (Fig. 3). On the same figure, the HDT's of the enzymes studied were arranged, as described above. The autogenous syntheses of ATCase, OTCase, and DHQase occurred in the same order, and at the same times relative to the increase in potential for sucrase synthesis, as did the replications of their genetic markers. The enzyme map appears to be colinear with the genetic map presented below it.

*Correlation of the increase in potential for sucrase synthesis with the doubling of the sucrase gene:* Although the order of enzyme synthesis appears to be the same as that

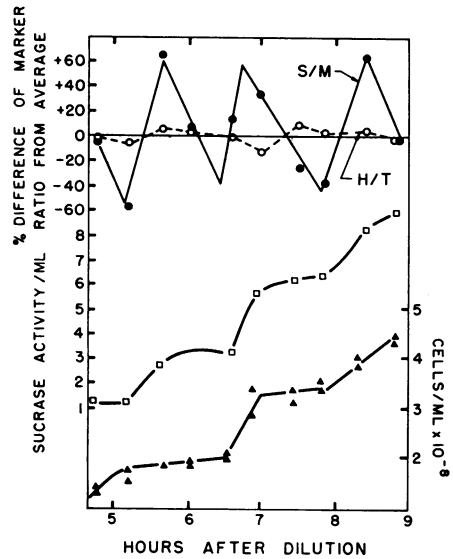


FIG. 4.—Correlation of the time of increase in potential to synthesize sucrase with the time of doubling of the sucrase gene. Open squares represent the potential of the culture, whose cell number is represented by closed triangles, to synthesize sucrase at various times during synchronous growth. At various times aliquots of the culture were taken for preparation of transforming DNA. This DNA was used to transform an auxotroph requiring methionine and unable to ferment sucrose. The ratio of transforming activities for these markers, relative to the average ratio, is plotted as closed circles. The corresponding determinations of histidine/tryptophan transforming ability, independently determined with the same DNA, are plotted as open circles.

of gene replication, the question remains as to whether the two processes are in phase. The following experiment suggests that the increase in *potential* to synthesize sucrase is in phase with doubling of the corresponding gene. The transformability of *B. subtilis* provides a means to measure the times at which specific genes double during the course of the cell cycle. For example, as the concentration of sucrase genes doubles with respect to the concentration of methionine genes in a synchronous culture, the ratio of sucrase transformants to methionine transformants would also be expected to double. The ratio of the two classes of transformants would, in fact, be expected to vary over a twofold range with a period equal to that of the cell cycle.

The ratio of sucrase to methionine transforming ability was determined for each of the DNA samples taken from a synchronous culture (Fig. 4). The percentage difference of each ratio from the average ratio for all determinations was plotted

TABLE 1

GENE MAPPING DATA

| Marker mapped | Genotype of recipient | Marker ratio* | | A Ratio corrected to methionine as base $\left(\frac{X_0}{Y_0} \times \frac{Y_0}{Meth_0}\right)$ | Position on enzyme map | B Genetic map position 0 Ade Meth 1 |
|---|---|---|---|---|---|---|
| Sucrase | *Suc⁻tryp₂⁻meth⁻* | $S_0/M_0$ | 1.98 | 1.98 | 0.0 | 0.013 |
| | | $S_0/T_0$ | 1.76 | 1.98 | — | 0.013 |
| *Tryp₂* | *Suc⁻tryp₂⁻meth⁻* | $T_0/M_0$ | 1.12 | — | — | 0.84 |
| | | | 1.10 | — | — | 0.86 |
| | | | 1.14 | — | — | 0.81 |
| | | | 1.09 | — | — | 0.87 |
| OTCase | OTC⁻*leu⁻tryp₂⁻* | $OTC_0/T_0$ | 1.09 | 1.21 | 0.73 | 0.72 |
| ATCase | *Tryp₂⁻ATC⁻hist₁⁻* | $ATC_0/T_0$ | 1.08 | 1.20 | 0.73 | 0.74 |
| *His₁* | " | $H_0/T_0$ | 1.25 | 1.39 | — | 0.53 |
| Dehydroquinase | DHQ⁻*tryp₂⁻* | $DHQ_0/T_0$ | 0.94 | 1.02 | 1.03 | 0.97 |
| | | | 0.87 | 0.97 | — | 1.02 |

* $\frac{(X \log/Y \log)}{(X \text{ stat}/Y \text{ stat})} = X_0/Y_0.$

against the time of sampling. These ratios, within the limits of error, were distributed around their average in a manner consistent with a twofold change in relative marker concentration during the course of each cell cycle, although too few points were obtained to permit the accurate drawing of a curve.

The proportion of sucrase markers increased once per cycle, at approximately the same time as did the rate of induced enzyme synthesis. This result is consistent with the hypothesis that replication of a specific marker on the *B. subtilis* genome is in phase with the increased potential for synthesis of the corresponding enzyme.

An error analysis of this experiment suggested that the variations in ratios that were obtained were much greater than would be expected by chance. In order to show this experimentally, the same DNA samples were used to transform a $his_2^-$ $tryp_2^-$ recipient. Since the latter two markers are linked,[10] the ratio of $his_2^+/tryp_2^+$ transformants would remain close to their average value at all times if the experimental procedure is valid. This indeed was the case (Fig. 4).

Thus, a correlation is seen between potential increase and gene doubling for sucrase. The question of the relation between autogenous increase and gene doubling is being investigated.

*Discussion.*—In a previous publication we suggested that the sequence of autogenous enzyme steps could provide a method for the mapping of their corresponding genes in cases where more conventional methods failed.[1] In the light of these results this method could seem to provide only a very approximate estimate of map position. The timing of changes in potential for enzyme synthesis could be expected to reflect the course of genetic events more precisely.

If the potential for a specific enzyme synthesis is a function of its gene dosage,[11, 12] the potential should change when the gene doubles. The order of change in synthetic potential of a set of enzymes would be expected to follow the order of the genes on the genome. This appears to be the case for a number of enzymes of *E. coli.*[13] The correlation of increased potential for sucrase synthesis with the increased transforming activity for the sucrase marker, which is reported here, provides direct support for this hypothesis.

The major question we hoped to answer was whether the order of autogenous steps of discontinuously synthesized enzymes shows a correspondence with the genetic order in the same manner as does synthetic potential. If the positions of autogenous steps were determined by a cyclic variation in repressor levels,[2] such a correspondence would be possible, though not necessary.

The results reported above indicate that such a correspondence does exist. On the average, the order of autogenous steps is the same as that of the corresponding genetic markers. However, the timing of enzyme steps seems to be subject to variation. The variation is great enough to allow reversals of position of autogenous steps whose average values are relatively close. Thus, histidase (average HDT = 0.54) and ATCase (average HDT = 0.73) had values in a particular cycle of 0.59 for histidase and 0.55 for ATCase. This suggests that there is no fixed relationship between the time of gene doubling and the time of autogenous synthesis of the corresponding enzyme. It is probable that the sequential synthesis of genes basically determines the order of autogenous steps, but that the exact timing of the steps is subject to other controls not strictly in phase with the replication of the genome.

In these experiments, periods of autogenous synthesis of enzymes are scattered throughout the entire cell cycle. Any simple relationship between gene dosage and enzyme synthesis would require that replication of the genome occupy the entire cell cycle.[14] Preliminary experiments indicate that the DNA synthesis in these cultures is completed in about half a cell cycle. The spacing of the points in Figure 4 suggests that a considerable part of the cycle must intervene between termination of chromosome replication (doubling of the *meth* marker) and initiation of the next round (doubling of the *suc* marker). The implications of this observation will be discussed more fully in a subsequent communication.

*Summary.*—The syntheses of five enzymes during the cell cycle of *B. subtilis* have been investigated using synchronous cultures. The autogenous syntheses of four of these and the change in potential to synthesize the fifth were rapid during only a portion of the cell cycle. The times of rapid synthesis could be arranged in an average linear order. This order was the same as that of the corresponding genetic markers for the four enzymes for which negative mutants were available. Although the average sequence of autogenous enzyme syntheses was in accordance with the genetic map, some variation was apparent, including, in particular cycles, occasional reversals in order of synthesis of relatively closely linked enzymes.

In addition, relative sucrase-transforming activity changed periodically during the cell cycle, and at approximately the same time as the potential for sucrase synthesis.

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