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Synthesis of Bacterial Flagella: Chromosomal Synchrony and Flagella Synthesis

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Synchronous cultures of *Bacillus subtilis* 168 M were obtained from light-density spores germinated at 46 C and grown at 37 C. This procedure synchronizes both cell division and chromosome replication. The chromosome synchrony was demonstrated by using transformation to measure changes in marker frequency during the cell cycle. The synthesis of two enzymes and of bacterial flagellar protein was also followed. All of the proteins were found to be synthesized continuously with an abrupt doubling in the rate of synthesis at a specific time in the cell cycle. The time at which the doubling occurred for each enzyme corresponded to the time at which the structural gene for the enzyme was replicated. The doubling of the rate of flagella synthesis corresponded to the time of replication of the *hisA1* gene. We conclude that the genetic locus for the factors involved in the rate-limiting steps in flagella synthesis are located on the genetic map near the *hisA1* locus.

Kuempel, Masters, and Pardee (4) analyzed the kinetics of enzyme synthesis during cell synchrony in terms of the potential and autogenous rate. They defined the potential as the maximum ability for enzyme synthesis and assumed it to be directly proportional to gene dosage. Therefore, under conditions where the potential for the synthesis of a specific enzyme can be measured directly, it provides information about the time at which the corresponding gene is replicated. A number of investigators have shown (2, 4, 5, 10) that under conditions of cell synchrony the timing of the transcription or translation of specific genes may be governed by their position on the genetic map.

As a result of the investigation of synthesis of flagella in several temperature-sensitive mutants, we found that it was possible to synchronize a culture of *Bacillus subtilis* 168 M by germinating spores at 46 C and shifting the culture to 37 C. The present communication describes some of the properties of this synchrony. Measurements of the kinetics of enzyme and flagella synthesis suggest that chromosome replication is synchronized and that the genes controlling rate-limiting steps in flagella synthesis are located on the chromosome near the *hisA1* locus.

MATERIALS AND METHODS

Organism. *B. subtilis* 168 M, an indole auxotroph transformed to wild type, was used as a source of spores for synchronous cultures. Spores were harvested after growth on AK medium no. 2 (BBL) for 1 day at 37 C and 3 days at 22 C, with cold, distilled water. They were washed free of medium with water, suspended in 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer containing 0.01 M ethylenediaminetetraacetate (EDTA; *p*H 8.1) and 200 μg of lysozyme per ml, and incubated for 30 min at 37 C. The spores were washed again in water, resuspended after the second wash to 100 mg (wet weight)/ml, and stored at 4 C.

The following double auxotrophs were used as recipients for transformation studies: BR 62 *purA16 trp-2* (requiring adenine and tryptophan), BR 19 *hisA1 trp-2* (requiring histidine and tryptophan), and BR 44 *leu-6 trp-2* (requiring leucine and tryptophan). These strains were constructed by B. Reilly in J. Spizizen's laboratory (Scripps Clinic and Research Foundation, La Jolla, Calif.).

Media. *B. subtilis* 168 M was grown on Spizizen's minimal medium (8) supplemented with 0.5% glucose and 0.1% acid-hydrolyzed casein. Transformations were carried out by the method of Spizizen (8).

Deoxyribonucleic acid (DNA) preparation. Samples (20 ml) taken from 0 to 35 min and 10-ml samples taken from 40 to 70 min during synchronous growth were delivered into an equal volume of ice-cold minimal medium containing 0.5% glucose. After washing twice in 0.2 M Tris buffer containing 0.01 M EDTA, *p*H 8.1, the cells were resuspended in 0.9 ml of the same buffer containing 200 μg of lysozyme (Worthington Biochemical Corp.) per ml. After incubation at 37 C for 5 min, 0.1 ml of Pronase (B grade, Calbiochem) containing 40 mg of enzyme/ml was added, and the preparations were incubated for 12 to 18 hr at

46 C. A 1-ml amount of 4 M NaCl was added, and particulate matter was removed by centrifugation. Each DNA preparation contained 3.8 to 4.5 μg of DNA/ml, based on an estimate of 1 μg of DNA/10^8 cells and assuming complete lysis. A 0.1-ml amount of a DNA preparation was used for each transformation experiment carried out in a total volume of 1.0 ml.

Marker normalization. Marker ratios were not normalized. Ratios presented are those obtained by dividing the number of recombinants for *his*, *ade*, or *leu* by the number of recombinants for *trp* and using for transformation DNA from a given time point during synchronized growth.

Spore preparation. Light and heavy density spores were separated by a modification of the procedure of Tamir and Gilvarg (9). A gradient was prepared consisting of 200 ml of 70% (v/v) Renografin-76 (Squibb Methylglucamine Diatrizoate, E. R. Squibb and Sons, Inc., New York, N.Y.), followed by 20 ml of 30% (v/v) Renografin-76. Spores collected as previously described were suspended in 15% Renografin at a concentration of 50 mg/ml (wet weight), and 6 ml of the suspension was layered on the gradient. Samples were centrifuged for 30 min at 16,000 rev/min in an SW25 Spinco rotor. Heavy-density spores pelleted, whereas light-density spores formed a band near the top of the 70% solution. The spores were collected and diluted 1:1 with cold, distilled water and washed three times with water by centrifugation at 15,000 rev/min for 5 min. Light-density spores were stored in distilled water at 4 C.

Synchronization. Light-density spores were suspended in Spizizen's minimal medium (8) containing 0.5% glucose and 0.1% acid-hydrolyzed casein to a final concentration of 2×10^9/ml and then incubated at 46 C for 12 to 16 hr with shaking. The culture was diluted 1:10 into the same medium preheated to 46 C and incubated for 75 to 90 min. The cells were centrifuged in the cold and diluted to 10^7 cells/ml (or about 20 Klett units with the 660-nm filter) in the same fresh medium preheated to 37 C. Upon continued incubation at 37 C with shaking, the first cell division occurred in 25 to 50 min after the transfer to 37 C.

Plate counts were performed as follows. Samples (0.1 ml) were added to 9.90 ml of minimal medium containing 0.5% glucose and 0.1 mg of Pronase per ml; the diluted sample was incubated for 5 min at 37 C; the sample was then further diluted and spread immediately on Tryptose Blood Agar Base plates (Difco). Omission of the 0.5% glucose resulted in some cell lysis, and omission of the Pronase resulted in much lower cell counts at the beginning of each cell cycle.

Flagella assay. The assay for flagellar antigens was carried out by using ^{125}I-antibody as previously described by Grant and Simon (3).

5-Dehydroquinase assay. 5-Dehydroquinase was assayed by the procedure of Mitsuhashi and Davis (6). Activity is expressed as units of enzyme where one unit catalyzes the formation of 1 nmole of dehydroshikimic acid per min.

Phosphoglucomutase (PGM) assay. A modification of the procedure of Pontis and Leloir (7) was used. One assay cuvette contained the following reagents in a final volume of 1 ml: 0.05 M Tris buffer, *p*H 8.0, containing 10^{-2} M Mg and 10^{-3} M EDTA; 1.0 unit of glucose-6-phosphate dehydrogenase (Sigma); 20 mM cysteine-hydrochloride neutralized to *p*H 7.0; 20 μM nicotinamide adenine dinucleotide phosphate (Calbiobiochem); 14 μM glucose-1-phosphate (Calbiochem); 36 μM glucose-1,6-diphosphate Calbiochem); 6.7 mM MgCl.

The extract was prepared by centrifuging the sample taken from a synchronized culture, suspending the pellet in the Tris buffer used in the assay containing 200 μg of lysozyme per ml, incubating for 5 min at 37 C, and then removing any particulate matter by centrifugation. Activity is expressed as units of enzyme where one unit splits 0.1 nmole of uridine diphosphate-glucose, which corresponds to $\Delta A_{340} = 6.3 \times 10^{-4}$/min.

RESULTS

Synchrony. When spore preparations were germinated at 46 C and then shifted to 37 C, the cells divided synchronously. However, the culture behaved heterogenously, i.e., the synchrony was not maintained for more than one generation. To obtain better synchrony, we examined the initial spore preparation. The spores were fractionated on a Renografin gradient (A. Evans, *personal communication*). Light-density spores formed a discrete band and heavy spores sedimented to the bottom of the tube. Figure 1 shows the results of attempts to obtain synchronized cultures with the purified spore preparations. Only the light-density spores gave rise to synchronized cultures. The cells obtained from the heavy-density spores grew exponentially.

We have been able to obtain this synchronization with a number of derivatives of the *B. subtilis* 168 strain, including tryptophan and lysine auxotrophs. Most of our work has been done with a 168 M prototrophic strain and synchrony has been consistently obtained. There was always a time lag after the cells were shifted to 37 C and before the first division. The lag was usually between 25 and 50 min. The time required for a full division cycle varies depending upon the strain. The generation time for the 168 M prototroph was 35 min.

Figure 2 shows the results of measurements of the rates of flagella and 5-dehydroquinase synthesis during cell synchrony. Both proteins are synthesized continuously; however, the rates of synthesis change abruptly at a specific time in the cell cycle. The rate of synthesis of flagellar protein doubles at 70 min and the rate of 5-dehydroquinase synthesis doubles at 90 min. One way to explain these specific changes is to assume that they reflect a doubling of the genes that correspond to each protein (1).

To test this explanation, the kinetics of PGM

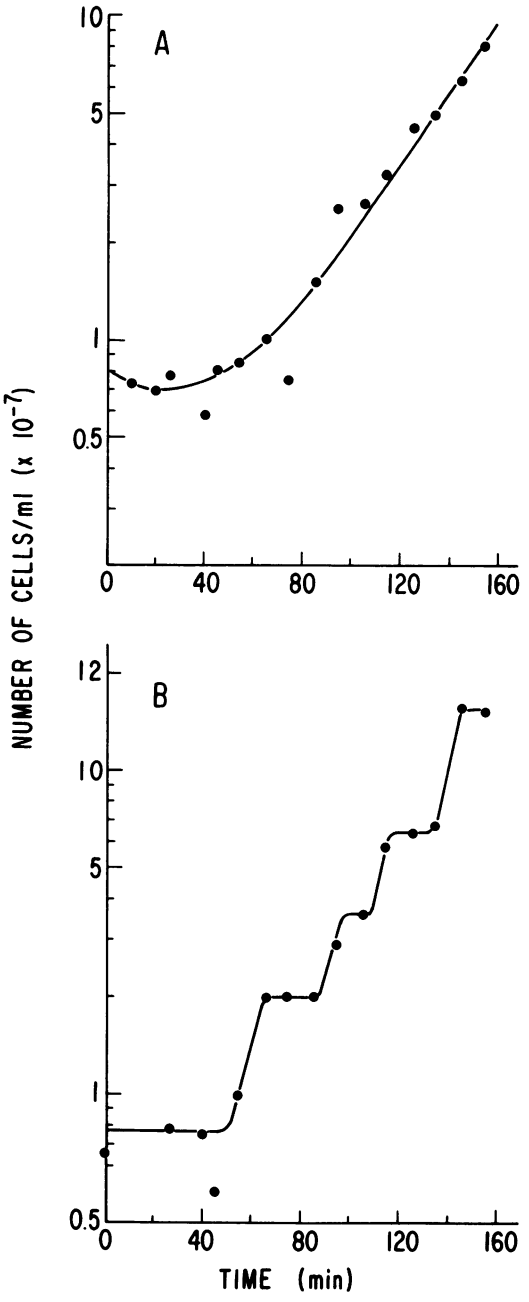

FIG. 1. *Application of the synchronization procedure to light- and heavy-density spores. (A) Heavy-density spores: (B) light-density spores.*

synthesis and the *hisA1* marker frequency were measured during the synchrony. We have shown by transduction (G. F. Grant and M. Simon, *submitted for publication*) that the genes that control flagella synthesis are linked to the *hisA1*

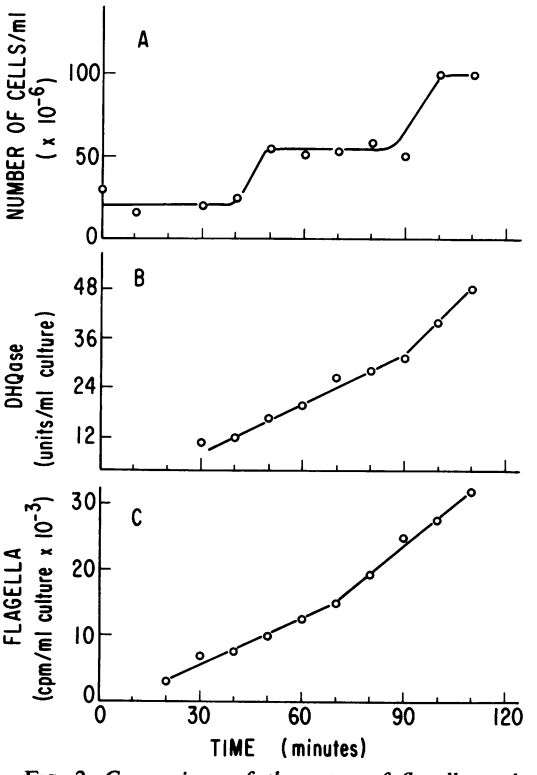

FIG. 2. *Comparison of the rates of flagella and dehydroquinase synthesis during cell synchrony. (A) Cell count; (B) dehydroquinase activity; (C) flagellar activity.*

gene and the structural gene for PGM (the *gtaC* locus). If the time at which the rate of flagella synthesis doubles corresponds to a doubling of the flagellar genes, we would also expect the *hisA1* marker frequency and the rate of PGM synthesis to double at the same time.

The results (Fig. 3) confirm this prediction: the rate of PGM synthesis, flagella synthesis, and the *hisA1* frequency all double between 30 and 35 min after incubation at 37 C.

These data taken together suggest that chromosomal replication is synchronous and that the changes in enzyme synthesis reflect this synchrony.

To follow chromosomal replication, double-auxotrophic recipients were transformed with DNA taken from cells at various times during synchronized growth. Three markers were followed: *purA16* which is near the beginning of the map, *hisA1* which is near the middle, and *leu-6* which is near the end (1). These were compared to the *trp-2* marker which maps in the terminal region of the chromosome. The ratio for the markers double abruptly (Fig. 4);

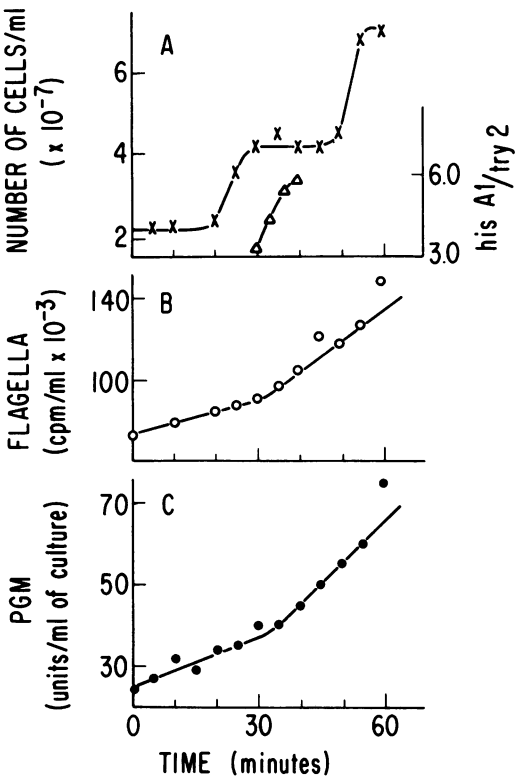

FIG. 3. *Comparison of the rates of flagella and phosphoglucomutase synthesis and hisA1 replication during cell synchrony. (A) ×, cell count; △, marker frequency. (B) Rate of flagella synthesis. (C) Rate of phosphoglucomutase synthesis.*

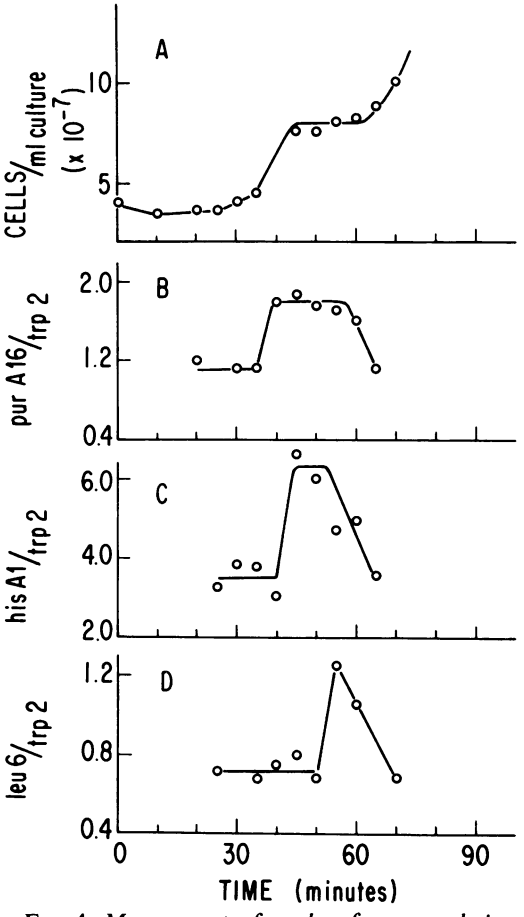

FIG. 4. *Measurement of marker frequency during cell synchrony. Samples were taken from a synchronized culture. DNA was prepared from each sample and then used to transform double auxotrophs. The marker ratios presented in B, C, and D represent the number of transformants for purA16, hisA1, or leu 6 divided by the number of transformants for trp 2. (A) Cell number; (B) ratio of purA16/trp 2; (C) ratio of hisA1/trp 2; (D) ratio of leu 6/trp 2.*

purA16 is replicated between 35 and 40 min, *hisA1* between 40 and 45 min, *leu-6* between 50 and 55 min, and finally *trp-2* between 55 and 65 min. The relative doubling time of the markers corresponds reasonably well to their position on the genetic map.

DISCUSSION

The efficacy of the synchronization procedure may result from a number of factors. The method involves spore germination, dilution of stationary-phase cells, and temperature shifts. All of these techniques have been used to synchronize cells. We have attempted to vary the procedure by changing the time of incubation, decreasing the concentration of the spore inoculum, and by omitting the interim growth period at 46 C. However, any significant change in the method eliminates the synchrony. This suggests that the entire process, including the initial light-density spore germination step, is required to obtain synchrony.

Although this communication utilizes the near doubling of marker ratios in order to show that chromosome replication is synchronized, it may also be seen that the doubling of each ratio during synchronized cell browth occurs at a time which closely corresponds to the position of the respective locus on the genetic map. The doubling of the ratios also agrees well with the replication sequence demonstrated in *B. subtilis* W23 (11). However, owing to the rather short generation time, this technique cannot be used for accurate mapping.

Although we have measured the kinetics of synthesis of only three proteins during the

synchrony, their behavior is similar. They are all synthesized continuously and their rate of synthesis doubles abruptly at a specific time during the cycle. This behavior suggests that enzyme synthesis is directly proportional to its potential, and in fact the time at which the rate of enzyme synthesis doubles both for 5-dehydroquinase and PGM corresponds to the time at which the corresponding gene is replicated. We may speculate that, since the cells are growing relatively rapidly with a division time of 30 min, gene products are being synthesized at near maximal rates. On this basis, the kinetics of flagella synthesis suggest that the genes involved in a rate-limiting step in flagella development are replicated at about the same time as the *hisA1* gene. We have shown, by transduction, that the flagellar genes are linked to the *hisA1* locus. The properties of the synchrony suggest that it will be useful for investigation of the mechanisms of DNA replication and cell division.

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