JOURNAL OF BACTERIOLOGY, Dec. 1969, p. 1316-1321
Vol. 100, No. 3

Initial Characterization of a Temperature-Sensitive Rod⁻ Mutant of *Bacillus subtilis*[1]

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Received for publication 6 October 1969

The general biological properties of a temperature-sensitive morphological mutant of *Bacillus subtilis* (168ts-200B) are described. At the restrictive temperature (45 C), cells grow as spheres which divide irregularly to form grapelike clusters. At the permissive temperature (30 C), the mutant grows as typical *B. subtilis* rods in short chains. A log-phase culture of rods (30 C) may be converted to spheres by transfer to 45 C. Reversion of spheres to rods occurs when the alternate temperature shift is made. Growth curves, deoxyribonucleic acid replication kinetics, and the morphology of mutant 168ts-200B are described.

Although there is currently widespread interest in the use of microbial systems to study aspects of the genetic control of morphology, analysis of bacterial morphology mutants has only recently been reported (1-3, 6, 9, 10). A rod⁻ mutant of *Bacillus subtilis* which grows as typical rods when the growth medium is supplemented with 0.8 M NaCl but which grows as cocci in unsupplemented medium has been described (8). In addition, some temperature-sensitive mutants of *B. subtilis* and *Escherichia coli* defective in deoxyribonucleic acid (DNA) synthesis develop abnormalities in morphology when grown at the restrictive temperature (4, 5, 7). This report describes the morphology, DNA kinetics, and growth curves of a temperature-sensitive mutant of *B. subtilis* which grows as typical rods at the permissive temperature (30 C) but which grows as irregular spheres at the restrictive temperature (45 C). Conversion of rods to spheres or reversion of spheres to rods occurs when a log-phase culture growing at either the permissive or restrictive temperature is shifted to the alternate temperature.

MATERIALS AND METHODS

Organism. The parent strain (designated 168ts⁺) is the indole⁻ thymine⁻ mutant of *B. subtilis* 168. Mutant 168ts-200B is a temperature-sensitive mutant derived from 168ts⁺.

Media. Minimal salts consisted of (in grams per liter): $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 14.0; KH_2PO_4, 6.0; sodium citrate·$2H_2O$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2. Minimal medium consisted of the above salts with the addition of glucose (0.5%), casein hydrolysate (0.2%), L-tryptophan (20 μg/ml), and thymine (20 μg/ml). Each component was autoclaved separately and added aseptically. Agar (15 g per liter) was added for plating. Trypticase Soy Agar (TSA, BBL) plus 20 μg of thymine per ml was also used for plating.

Spores. Fresh potato extract was obtained from 200 g of diced potatoes by heating in 1 liter of water at 100 C for 5 min and filtering. Tryptone (20 g) and yeast extract (2 g) were added to the extract, and the *p*H was adjusted to 7.2. The volume was brought to 2 liters, 30 g of agar was added, and the solution was autoclaved. Thymine at a final concentration of 20 μg/ml was added aseptically. Phase-bright spores were harvested and purified by the method of Yoshikawa (11).

Isolation of 168ts-200B. Spores of *B. subtilis* 168ts⁺ were heat-shocked for 15 min at 70 C and grown overnight (ON) at 30 C in minimal medium with shaking. A sample was diluted 100-fold in fresh medium and incubation continued for 4 hr. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) at a concentration of 50 μg/ml was added and incubation continued for 20 min. The cells were filtered and washed four times with minimal salts and then grown ON in minimal medium at 30 C. The culture was diluted and spread on TSA plus thymine plates to obtain approximately 100 colonies per plate. After 15 hr at 30 C, the plates were replicated on plates of the same composition prewarmed at 45 C. After 5 hr of incubation at 45 C, master and replica plates were scored and temperature-sensitive colonies were selected for further studies. Markers and temperature-sensitivity were rechecked before adding the new mutants to the collection.

Kinetics of protein and DNA synthesis. Cells were grown in liquid minimal medium with bubbling aera-

[1] Presented in part at the 69th Annual Meeting of the American Society for Microbiology, Miami, 1969. This work was done in partial fulfillment of the requirements for the Ph.D. degree in the University of Maryland by Robert J. Boylan.

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tion. The growth temperatures were 30 and 45 C. Measurements with a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.) at OD_{660} served as a measurement of protein synthesis. The incorporation of thymine-2-^{14}C (56.5 mc/mmole; New England Nuclear Corp., Boston, Mass.) into components insoluble in cold 10% trichloroacetic acid was used as a measurement of DNA synthesis. Samples (0.1 ml) were removed from the growing cultures at the indicated times and added to 8 ml of trichloroacetic acid. The acid-insoluble components were collected on HA filters (Millipore Corp., Bedford, Mass.) and washed twice with additional trichloroacetic acid. The dried filters were counted in a gas flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

Morphology. Cells were sampled into 1% formaldehyde. Wet mounts were examined with a Wild phase-contrast microscope fitted with a Wild high-resolution oil immersion objective.

RESULTS

Morphology. The morphology of *B. subtilis* 168ts-200B grown at restrictive and permissive conditions is shown in Fig. 1 and 4. Spores of 168ts-200B germinated and grown at 45 C (Fig. 1a–d) emerge as spherical cells which continue to divide in this form until clusters of up to 20 to 30 spheres are produced. When a log-phase culture of spherical cells at 45 C is shifted to the permissive temperature (30 C) and incubation continued (Fig. 2a–d), a reversion to the bacillary form occurs. About 2 hr after transfer to 30 C (Fig. 2a), a number of fat U-shaped cells are visible within the mass. This is the early appearance of spheres which are able to give rise to rod-shaped progeny. The fat U-shaped cells appear to elongate gradually with the first division occurring in or near the middle of the U-cell. As growth continues, the terminal cells often remain attached in some manner to a structure within the body of the main cell mass. Thus a high proportion of "loop-shaped" arrangements of rods in chains around the periphery of the main cell mass is produced.

If a young culture (45 C) consisting of single spherical cells emerging from each spore (Fig. 1a) is transferred to 30 C, virtually all of the spheres are converted to rods. On the other hand, in older cultures (45 C) of spheres transferred to 30 C, not all spheres revert to rods. In masses of spheres (Fig. 1d), it is impossible to determine

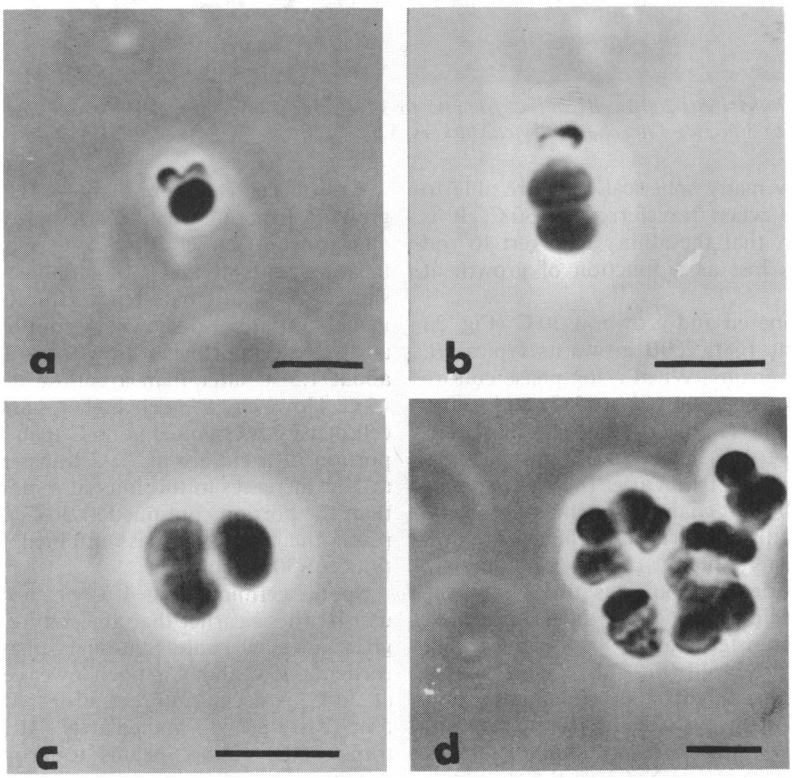

FIG. 1. *B. subtilis 168ts-200B cells grown from spores at 45 C for (a) 1.5 hr, (b) 2.5 hr, (c) 3.5 hr, and (d) 5 hr. Markers, 5 μm.*

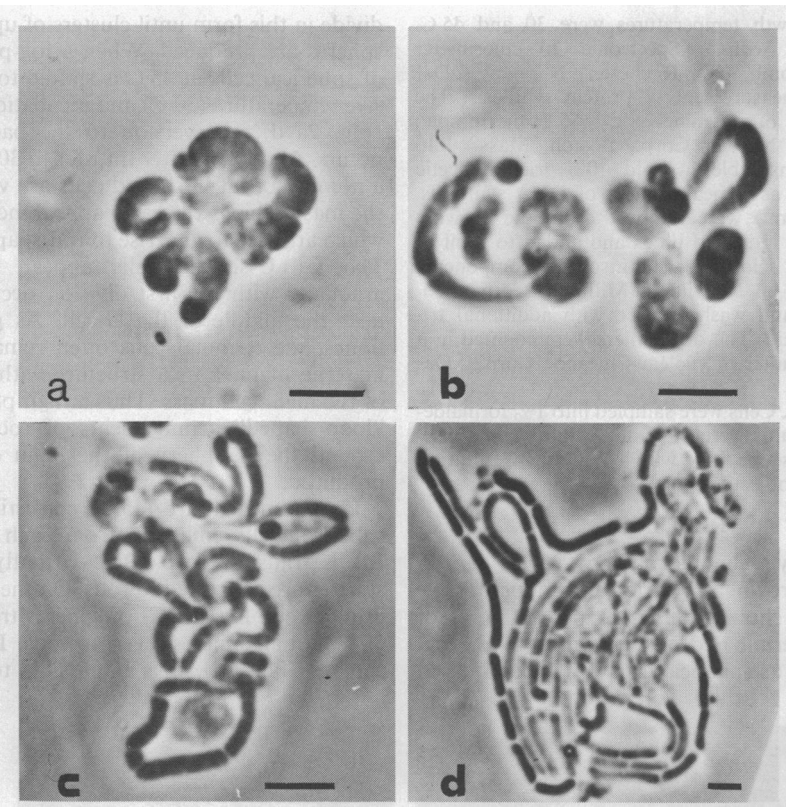

FIG. 2. *B. subtilis 168ts-200B cells shifted from 45 to 30 C after 5 hr growth from spores and grown at 30 C for (a) 2 hr, (b) 3 hr, (c) 4 hr, and (d) 5 hr. Markers, 5 μm.*

accurately how many spherical cells are able to revert to rods when transferred to 30 C. It is clear, however, that the ability to revert to rod morphology is lost as a function of growth at 45 C.

When germinated and grown at 30 C (Fig. 3a and b), mutant 168ts-200B grows as typical *B. subtilis* rods in chains. When a log-phase culture growing at 30 C is transferred to 45 C and incubation is continued (Fig. 4a–d), a gradual conversion of rods to spheres occurs. Slight swellings are apparent within 30 min after transfer in about 50% of the cells in a chain. The remainder of the rods are gradually converted to spheres. About 2 hr after transfer, many chains of complete spheres are evident. About this time, however, the spheres begin to divide in different planes. Three hr after transfer, masses of spheres growing in clusters are produced (Fig. 4d).

Spores of mutant 168ts-200B germinated and grown at 45 C in hypertonic environments continue to grow as spheres. For example, supplements of 1 M sucrose, 1 M NaCl, or 0.5 M sodium succinate added to the growth medium have no obvious effect on sphere morphology.

Growth curve. Figure 5 shows the patterns of growth of mutant 168ts-200B spores germinated and grown at either 30 or 45 C. Optical density measurements (OD_{660}) of cultures which were shifted to the alternate temperature after 5 hr of incubation are also shown. A culture germinated at 30 C does not begin growth for about 3 hr—about 1.5 hr later than a culture germinated at 45 C. However, a much higher stationary-phase cell density is obtained at 30 C than at 45 C. The portion of a culture at 30 C transferred to 45 C at 5 hr increases in turbidity at a more rapid rate than the portion continued at 30 C, but does not reach the final cell mass attained by the continuous (30 C) culture.

Spores germinated at 45 C begin macromolecular synthesis earlier than the culture at 30 C, but grow at a slower rate. Stationary phase is reached earlier at 45 C and at a much lower cell mass than at 30 C. A 45 C culture transferred to 30 C at 5 hr ceases growth immediately. At 30 min after transfer the culture begins to drop in optical density. This drop continues for about 1 hr, after which a slow increase in cell mass resumes.

DNA synthesis. Figure 6 represents the kinetics

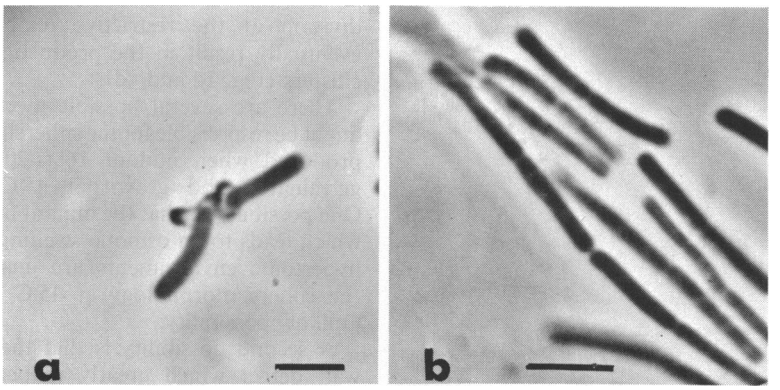

FIG. 3. *B. subtilis 168ts-200B cells grown from spores at 30 C for (a) 3.5 hr and (b) 5 hr. Markers, 5 μm.*

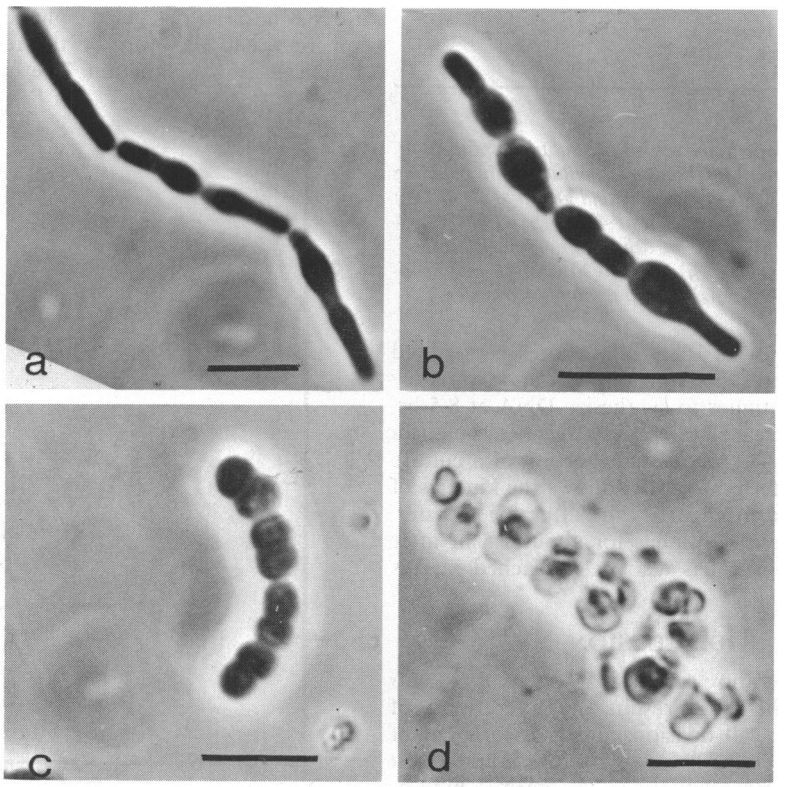

FIG. 4. *B. subtilis 168ts-200B cells shifted from 30 to 45 C after 5 hr growth from spores and grown at 45 C for (a) 30 min, (b) 1 hr, (c) 1.5 hr, and (d) 3 hr. Markers, 5 μm.*

of DNA synthesis of the cell population whose growth curves are shown in Fig. 5. DNA synthesis begins about the same time as the first noticeable increase in turbidity of the growing cell populations. Incorporation of ^{14}C-thymine at 45 C begins at about 2 hr and increases linearly between the 3rd and 8th hr. ^{14}C-thymine incorporation continues for about 2 hr after the last detectable increase in optical density. A culture of spheres transferred from 45 to 30 C continues to synthesize DNA for 1 hr after transfer even though growth apparently stops when the cells are shifted to 30 C. A lag in DNA synthesis for 30 min follows, after which synthesis resumes about the same time growth is resumed in the cells.

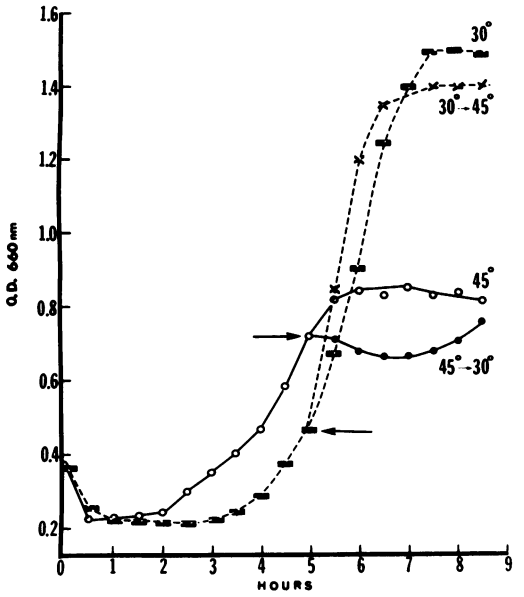

FIG. 5. *Growth curves of B. subtilis 168ts-200B. Cells were grown in minimal medium with bubbling aeration. (▬) Cells germinated and grown at 30 C. (○) Cells germinated and grown at 45 C. (×) Cells transferred from 30 to 45 C at 5 hr and continued at 45 C. (●) Cells transferred from 45 to 30 C at 5 hr and continued at 30 C. Arrows indicate points of transfer.*

A population of 168ts-200B spores germinated at 30 C initiates DNA synthesis at approximately 3 hr and continues to synthesize DNA at 8.5 hr, although increase in cell mass reaches a plateau at 7 hr. DNA synthesis in a portion of a culture (30 C) transferred to 45 C follows the kinetics of growth during the early time after the transfer. DNA synthesis continues for about 1.5 hr after stationary phase has also been reached in this population.

DISCUSSION

The temperature-sensitive defect of *B. subtilis* mutant 168ts-200B results in alterations of morphology, cell division, and gross amount of protein synthesis. These alterations could result from a thermosensitivity in the normal biosynthesis or maintenance of some component of the cell wall such as the mucopeptide. Electron microscopic examination of the mutant grown at 45 C clearly demonstrates anomalies in cell wall structure (report with R. Cole, *in preparation*). A weakening or disorganization of the rigid structure of the bacterial surface could lead to a spherical or irregular shape with a consequent distortion of the division septa. This in turn would result in the production of daughter cells at irregular positions. Continued growth and division at the restrictive temperature would eventually result in the production of grapelike clusters (Fig. 1d and 4d).

There are several possible mechanisms which might be responsible for the spherelike morphology produced when mutant 168ts-200B spores are germinated and grown at 45 C (Fig. 1a–d). One possibility is that the mutant has a wall defect which leads to an osmotic swelling. The fact that hypertonic environments are unable to reverse the sphere morphology at 45 C makes this an unlikely possibility.

A second possibility is that the mutant has a wall defect which greatly reduces the rate of linear wall synthesis or polymerization at 45 C. As new proteins and other macromolecules are synthesized at a normal rate, the cells would be forced into an irregular spherical shape. The sphere would therefore be the result of an unbalanced growth situation. The fact that protein

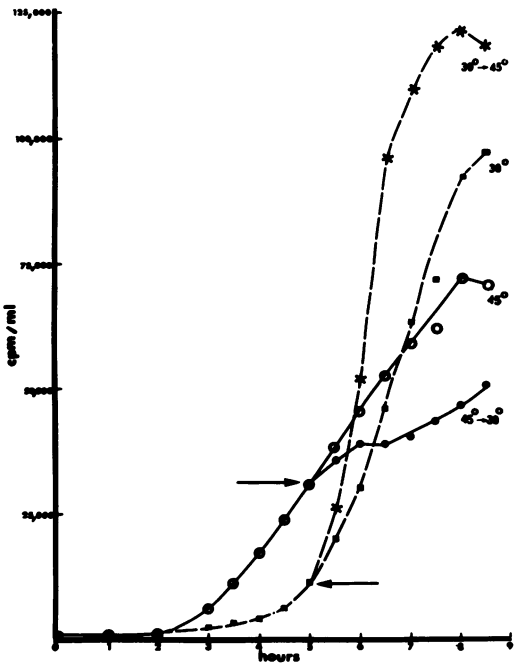

FIG. 6. *Kinetics of DNA synthesis during germination and growth of spores of B. subtilis 168ts-200B at both permissive and restrictive temperatures. Spores were germinated in minimal medium with 0.625 μc of thymine-2-^{14}C and 10 μg of ^{12}C-thymine per ml. Each tube was sampled at various intervals for acid-insoluble radioactivity. (▬) Cells germinated and grown at 30 C. (○) Cells germinated and grown at 45 C. (×) Cells transferred from 30 to 45 C at 5 hr and continued at 45 C. (●) Cells transferred from 45 to 30 C at 5 hr and continued at 30 C. Arrows indicate points of transfer.*

synthesis at 45 C is required for spheres to be produced is compatible with, although not proof of, this latter possibility.

The total conversion of rods to irregular spheres obtained when log-phase cells are transferred from 30 to 45 C (Fig. 4a–d) suggests that both existing and newly synthesized wall structures become disorganized at the restrictive temperature. The spherelike cells produced after a period of growth at 45 C are shorter and fatter than the original rods. Although the cells do not elongate properly, cross walls continue to be formed at 45 C. Eventually the orientation of these cross walls becomes irregular, and grapelike clusters of different sized cells are produced. The primary event in a rod to sphere conversion is probably the disorganization of rigid cell wall structure. It is not clear at present whether this is caused by a thermolabile wall structure per se or the activation at high temperature of some hydrolytic enzymes capable of attacking wall structure.

Clones of spherical cells produced by germination and growth of mutant 168ts-200B at 45 C are capable of reversion to the rod morphology when grown at 30 C (Fig. 2a–d). Not all spheres revert to the bacillary form however. The proportion of nonreverting spheres increases with longer periods of incubation at 45 C until finally, in stationary phase, the spheres are no longer able to revert. A clone of log-phase spherical cells (Fig. 2a) transferred to 30 C contains several cells able to revert to rods. Clones of this size also usually contain a number of nonreverting cells. The reversion process consists of synthesis of a newly organized wall, lengthening of the sphere into a U-shaped rod, followed by the production of properly oriented cross walls. The continued growth and division of these cells eventually produce a microcolony of rod cells. At the center of the microcolony there often remains a mass of spherelike cells unable to undergo reversion and to which the rods remain attached. These findings indicate that, despite the disorganized growth and division produced at 45 C, some cells are present which contain an intact genome and all cellular structures necessary for resumption of the normal growth pattern at 30 C.

The patterns of growth and DNA replication have been studied in mutant 168ts-200B growing under restrictive or permissive conditions and during transition from one environment to the other. The data (Fig. 5 and 6) indicate that spores germinated and grown at 30 or 45 C initiate DNA synthesis at about the time the turbidity of the culture begins to increase. The apparent linear rate of DNA synthesis observed in spherical cell clones might possibly be a secondary limitation resulting from the abnormal growth pattern. The initiation of DNA replication and segregation of daughter DNA molecules to daughter cells are known controlling mechanisms governing the rate of DNA replication. Both of these processes in normal cells are believed to be related to events taking place at the surface of the bacterial cell. Cells of abnormal morphology and growth pattern (168ts-200B grown at 45 C) might therefore be expected to display anomalous patterns of DNA replication and segregation as well. Our data demonstrate that DNA synthesis can initiate and proceed in cells of 168ts-200B grown at 45 C. We are currently in the process of an autoradiographic analysis of the segregation pattern in spherical cells of this mutant.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service training grant DE-00088-08 from the National Institute of Dental Research to R.J.B. and National Science Foundation grant GB-6651 to N.H.M.

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