JOURNAL OF BACTERIOLOGY, Feb. 1973, p. 985-998

Vol. 113, No. 2
Printed in U.S.A.

Temperature-Sensitive Divisionless Mutant of *Bacillus subtilis* Defective in the Initiation of Septation

XANDRA O. BREAKEFIELD[1] AND OTTO E. LANDMAN
Department of Biology, Georgetown University, Washington D.C. 20007

Received for publication 18 September 1972

A temperature-sensitive divisionless mutant of *Bacillus subtilis* 168, *tms-12*, is shown to be defective in an early step in septum formation at the restrictive temperature. The nature of this defect has been studied by comparing the growth and composition of mutant and wild-type (*tms-12*$^+$) cells at the restrictive (48 C) and permissive (34 C) temperatures. At 48 C, *tms-12* cells grow as nonseptate, multinucleate filaments. Filamentation does not appear to be a result of alterations in properties of the cell wall, since the ratio of mucopeptide to teichoic acid, the autolytic activity, and the ability of the walls to protect cells against osmotic shock are comparable in *tms-12* filaments and *tms-12*$^+$ bacilli grown at 48 C. Synthesis of deoxyribonucleic acid and the segregation of nucleoids also proceed normally during filamentation. The synthesis of membrane, however, is delayed during filamentation of *tms-12*. No gross alterations were observed in the protein or lipid composition of membranes isolated from mutant filaments. Septum formation resumes when filaments are returned to 34 C and appears to be associated with an increased synthesis of membrane. The occurrence of septa was monitored both by microscopic observation of cross walls and by assays of the number of viable protoplasts released from bacillary filaments upon removal of the cell wall. Septation recovery can be blocked by inhibitors of ribonucleic acid and protein synthesis added during, but not after, the first 7 min of recovery at 34 C. By contrast, inhibition of deoxyribonucleic synthesis does not block recovery.

Cell division in *Bacillus subtilis* is effected by the formation of a centrally positioned septum (10, 17). The septum consists both of a membraneous septum and of a cross wall and serves to partition the parent bacillus into two daughter cells. Septation precedes the physical separation of daughter bacilli (37). The manner in which septation is related causally, temporally, and spatially to other events of bacterial growth remains unclear; the evidence to date indicates that it may be linked with nuclear replication (12, 13, 20), with cell wall synthesis (39, 41; M. L. Higgins and G. D. Shockman, CRC Critical Rev. Microbiol., *in press*), or with increases in cell volume (15, 24, 32).

In the present study of septation, we have used a temperature-sensitive septationless mutant of *B. subtilis*, *tms-12*, originally described by Copeland and Marmur (14). This mutant is blocked at the restrictive temperature (48 C) in a process necessary for the initiation of septation, but is able to complete the formation of septa which had been initiated previously at the permissive temperature (34 C). Growth of the mutant at 48 C produces filaments. These filaments begin to septate in an almost synchronous manner when transferred to 34 C. The mutant thus permits analysis both of a particular block in septation and of the sequential steps in septum formation. Neither wall synthesis nor nuclear replication or segregation appear to be altered during filamentation. Septation recovery proceeds in the presence of inhibitors of deoxyribonucleic acid (DNA) synthesis. However, ribonucleic acid (RNA) and protein synthesis are required early in recovery to overcome the mutational block in septum initiation; subsequently, septum formation can proceed in the absence of ongoing RNA or protein synthesis. During filamentation and recovery of the mutant, the rate of membrane

[1] Present address: Laboratory of Biochemical Genetics, Heart and Lung Institute, National Institutes of Health, Bethesda, Md. 20014.

synthesis varies in concert with inhibition or acceleration of septation. (This work was carried out by X.O.B. in partial fulfillment of the requirements for the Ph.D. degree, Georgetown Univ., Washington; a summary of this work was presented at the 71st Annual Meet. Amer. Soc. Microbiol. [Furano and Landman, Bacteriol. Proc., p. 24, 1971].)

MATERIALS AND METHODS

Bacterial strains. The temperature-sensitive septationless strain used in these studies, *B. subtilis* 168 *purA16 leu-8 metB5 tms-12*, was obtained from J. C. Copeland. This strain was isolated by Copeland and Marmur from strain Mu5u8u16 by ethylmethane sulfonate mutagenesis (14). The *tms-12* mutation was localized in the *leu-8 metB5* region near the terminus of the *B. subtilis* chromosome by these authors; it behaves as a single mutation (or as closely linked sites) in transformation. The apparent conservation of nucleotide sequence homologies among the *tms-12* loci of the *Bacillaceae* during the evolutionary diversification of this family suggests that the *tms-12* gene controls an important biological function (14). An isogenic strain which septated normally at the restrictive temperature was obtained in our laboratory by transforming *tms-12* with DNA from our strain 435 (*trp thy*) and selecting for colonies formed on nutrient agar (Difco) at 48 C. This control strain, *tms-12*$^+$, retained the auxotrophic markers *purA16*, *leu-8*, and *metB5*. Strains were stored as spores at 4 C in water. Spore stocks were prepared according to the method described by Anraku and Landman (4).

Definitions. Terms used traditionally to describe the morphological forms of dividing *B. subtilis* must be defined more precisely if they are to serve in a detailed discussion of septation and daughter cell separation. Thus, "bacillus" as used in this paper refers to a wall-bounded colony-forming unit (CFU) which may or may not contain cross walls within it. By "filament" we mean an elongated bacillus. Daughter cells within a bacillus will be termed "individual cells" if they were distinguished by microscopic examination of wall-stained bacilli, each individual cell being bounded by a cross wall or a bacillary end, or both. By contrast, daughter cells detected following wall removal as L colony-forming units (26) will be called "protoplasts."

Media. All media and solutions were prepared in distilled, deionized water. Solutions were autoclaved separately at 20 psi for 15 min unless otherwise stated.

To prepare 1 liter of enriched minimal medium (modified from Copeland and Marmur [14]), 2 g of $(NH_4)_2SO_4$, 14 g of K_2HPO_4, 6 g of KH_2PO_4, and 1 g of sodium citrate·$2H_2O$ were dissolved in 886 ml of water. Then the following were added: adenine (30 mg in 60 ml of water), L-leucine (30 mg of in 3 ml water), L-methionine (30 mg in 3 ml of water), L-glutamic acid (30 mg in 1.5 ml of water, adjusted to *p*H 7.0 with KOH and filter-sterilized), L-asparagine (30 mg in 6 ml of water), D-glucose (5 g in 20 ml of water), acid-hydrolyzed casein (BBL; 200 mg in 10 ml of water) and $MgSO_4 \cdot 7H_2O$ (200 mg in 10 ml of water).

Osmotically stabilized enriched minimal medium is identical to enriched minimal medium described above, except that the salts solution is prepared in 686 ml of water and 200 ml of a 2.5 M sucrose solution is mixed with it on the day of use.

For dilution fluid, a solution of salts as in enriched minimal medium is prepared in 990 ml of water; $MgSO_4 \cdot 7H_2O$ (200 mg in 10 ml of water) is added to it.

Osmotically stabilized dilution fluid was prepared fresh on the day of use by mixing 70 ml of salts solution (see enriched minimal medium described above; salts dissolved in 700 ml of water) with 30 ml 2.5 M sucrose, 0.1 ml of 5×10^{-3} M Na_2EDTA, and 0.1 ml of 1 M $MgCl_2$.

To prepare 1 liter of osmotically stabilized plating medium (modified from Landman and Halle [26]), the following solutions were cooled to 50 C after autoclaving and then mixed in the order shown with gentle agitation: (i) 8 g of agar (Difco) and 20 g of gelatin (BBL.) melted in 530 ml of water by heating at 90 C for about 1 hr; (ii) 2 g of $(NH_4)_2SO_4$, 3.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, and 1 g of sodium citrate·$2H_2O$ in 100 ml of water; (iii) 250 ml of 2.0 M sodium succinate (*p*H 6.8–7.2); (iv) D-glucose, adenine, L-leucine, and L-methionine added as in enriched minimal medium described above; and (v) $MgCl_2 \cdot 6H_2O$ (200 mg in 10 ml of water).

Chemicals. Penicillin-G, actinomycin-D, and chloramphenicol were obtained from Parke, Davis and Co., and puromycin and D-cycloserine were from Nutritional Biochemicals. Pentachlorophenol (PCP) (Eastman Kodak) was dissolved in 95% ethanol; nalidixic acid (Calbiochemicals) was dissolved in water neutralized with KOH. 6-(*p*-hydroxyphenylazo)-uracil (HPUr) was a gift of Neil C. Brown, University of Maryland, Baltimore. Lysozyme, trypsin, and deoxyribonuclease were products of Worthington Biochemical Co.

Growth of cells. For liquid growth of bacilli, either enriched minimal medium or osmotically stabilized enriched minimal medium was used: when determining viable counts, the cells were diluted into dilution fluid or into osmotically stabilized dilution fluid, respectively. Both protoplast and bacillary CFU were assayed on osmotically stabilized plating medium. L colonies were counted after 3 days of incubation at 30 C.

For experimentation, spore stocks were inoculated onto osmotically stabilized plating medium and incubated overnight at 34 C. Cells were suspended in liquid medium to a density of approximately 10^7 bacilli/ml and then incubated in a water bath shaker at 34 C until the population density, measured as optical density at 660 nm (OD_{660nm}), showed a rate of logarithmic increase corresponding to a generation time of 35 min. The density of the cultures was maintained at 10^7 to 10^8 CFU/ml by periodic dilution with prewarmed medium.

The survival of mutant cells at 48 C was found to be dependent both on the length of exposure to this temperature and on the nutritional content of the

medium. For maximum survival, log-phase cultures were exposed to 48 C at an initial population density of 2×10^7 to 5×10^7 CFU/ml. Viable cell numbers were determined by plating cells on a "shift-down" medium, that is, one not supplemented with hydrolyzed casein. Logarithmic growth of the mutant at 48 C for more than 2 hr led to clumping and death of the filaments.

Preparation of protoplasts and determination of the yields of viable protoplasts per bacilli. Protoplasts were obtained by removing the cell walls of bacilli grown in osmotically stabilized enriched minimal medium. The cell wall was removed by incubating the bacilli without shaking at 34 C for 60 min in osmotically stabilized dilution fluid containing 300 μg of lysozyme/ml and 10^{-3} M NaN_3 (protoplasting fluid). The number of viable protoplasts was determined as the number of L CFU (26). When assaying the yield of protoplasts per bacillus or per filament, duplicate samples were removed from the culture, one sample was diluted and plated directly, and the other was diluted 1/100 into the protoplasting fluid and after wall removal plated as protoplasts.

Staining of cell walls and determination of the average length of individual cells. When staining cell walls, bacilli were mixed with PCP and NaCl to final concentrations of 10^{-3} M and 1.5 M, respectively. One drop each of this suspension and of chloroform was smeared onto a slide and dried in air. At the time of microscopic examination, one drop of 0.02% aqueous crystal violet was placed on the smear. Bacilli were examined using a Zeiss phase microscope equipped with an eyepiece micrometer. The total length and the number of cross walls per bacillus were determined for at least 30 bacilli in each sample. The average length of these individual cells was determined as the total length of the bacilli divided by 1 plus the total number of cross walls observed.

Biochemical determinations. Protein was determined by the method of Lowry et al. (30), phosphorus by the method of Ames and Dubin (3) as modified from Chen et al. (11), and hexosamine by the method of Rondle and Morgan (41).

Isolation, autolysis, and hydrolysis of cell walls. Logarithmically dividing bacilli in 500 ml of enriched minimal medium were incubated either at 48 C for 80 min or at 34 C to an equivalent final OD_{660nm}. Cultures were harvested by adding them to centrifuge bottles on ice containing one-quarter the culture volume of frozen potassium phosphate buffer (0.03 M, *p*H 6.7) and 4×10^{-4} M PCP. Cells were kept at 4 C and washed twice by centrifugation at 10,000 $\times$ *g* for 10 min with 500 ml of phosphate buffer containing 10^{-4} M PCP. The final cell pellet was resuspended in 10 ml of phosphate buffer containing 0.5 M sucrose, frozen in an ethanol-dry ice bath, and stored at −20 C.

For the isolation of walls, cells were thawed and sonicated in a salt ice bath for 5 to 10 min using a Branson model S-75 sonic oscillator at a maximum setting until microscopic examination showed that 99% of the cells had been broken. Unbroken cells were collected by centrifugation at 1,000 $\times$ *g* for 3 min and discarded. Wall fragments remaining in the supernatant were collected by centrifugation at 10,000 $\times$ *g* for 10 min and washed twice with 50 ml of phosphate buffer. The pellet of walls was stored at −20 C.

The autolytic activity present in wall samples was measured in the manner described by Young (46). Walls were resuspended in sodium carbonate buffer (0.02 M, *p*H 9.6) to an OD_{600nm} of 0.20 and incubated at either 34 or 48 C for 1 hr. At 5- to 10-min intervals the decrease in OD_{600nm} was noted.

When determining the content of phosphorus and hexosamine, wall samples were first resuspended in 2 ml of water and heated at 100 C for 15 min. They were then washed three times with equal volumes of water. The wall pellet was hydrolyzed in 1 ml of 6 N HCl in a glass ampoule at 100 C for 16 hr. The hydrolyzed samples were evaporated under vacuum over KOH pellets and then resuspended and reevaporatd three times with equal volumes of water. The ratio of mucopeptide to teichoic acid in walls of *B. subtilis* has been determined to be stable over a range of growth phases by Boyland and Ensign (8).

Preparation of membranes. Membranes were isolated from cells grown in enriched minimal medium by a procedure modified from those of Kaback (23) and Patch and Landman (36). Bacilli were grown and harvested as described above for the isolation of walls. Protoplasts were prepared from these bacilli by incubation in 50 ml of protoplasting fluid. The protoplasts were lysed by rapid mixing with 1 liter of tris(hydroxymethyl)aminomethane (Tris) buffer (0.025 M Tris-hydrochloride, *p*H 7.5) at room temperature. Deoxyribonuclease was added to a final concentration of 20 μg/ml, and the suspension was incubated at room temperature for 20 min. Subsequent procedures were performed at 4 C. Membranes were collected by centrifugation at 10,000 $\times$ *g* for 45 min. Membrane samples were washed three times with 50-ml volumes of Tris buffer by centrifugation at 55,000 $\times$ *g* for 20 min. The final pellet was thoroughly dispersed in 2 ml of this buffer and divided into 2 parts. One part was mixed with an equal volume of 0.4% sodium lauryl sulfate (SLS) prior to determination of protein. The other part was assayed for phosphorus content.

Isotopic measurement of mucopeptide and protein. Net syntheses of mucopeptide and protein were simultaneously measured using the cell fractionation technique of Park and Hancock (35). Exponentially growing cultures in enriched minimal medium were prelabeled with L-^{3}H-alanine 2.5 μg/ml (0.3 mCi/mmole; International Chemical and Nuclear Corp.) at 34 C for 60 min. At appropriate intervals thereafter, 5-ml samples were removed and cells were precipitated in 5% cold trichloroacetic acid. The fractionation procedure consisted of extraction of lipids with ethanol, extraction of nucleic acids and teichoic acids with hot 5% trichloroacetic acid, and digestion of proteins with trypsin. The remaining residue consists primarily of mucopeptide. Following the ethanol extraction, the precipitates were collected on Whatman no. 3 filter-paper discs, and subsequent extractions, digestions, and washings

were done on the discs. Radioactivity was assayed using a scintillation fluid consisting of 200 mg of 1,4-bis(2-[5-phenyloxazolyl]) benzene and 5 g of 2,5-diphenyloxazole dissolved in 1 liter of toluene. When L-^{3}H-phenylalanine was used at a similar concentration and specific activity, 95% of the label was found in the protein fraction and 5% in the mucopeptide fraction.

Isotopic measurement of lipid synthesis. The net synthesis of lipid was measured as the incorporation of ^{3}H-sodium acetate into the cellular lipid fraction. This fraction was obtained by a chloroform-methanol extraction procedure described by Mindich (33) as modified from Bligh and Dyer (7). Exponentially growing cultures in enriched minimal medium were prelabeled with ^{3}H-sodium acetate, 100 mg/ml (266 mCi/mmole), for 60 min at 34 C. At appropriate intervals thereafter, samples containing 10^8 to 2×10^8 CFU were removed, mixed with PCP, HCl, and sodium acetate at final concentrations of 10^{-3} M, 10^{-3} M, and 0.5 g/ml, respectively, and held on ice for 5 to 15 min. The cells were washed twice by centrifugation at 4 C with 10 ml of potassium phosphate buffer (0.1 M, *p*H 7.5) containing 10^{-3} M $MgCl_2$ and 10^{-4} M PCP. The pellets were then extracted with 2 ml of chloroform, 2 ml of methanol, and 1 ml of water. A sample of the chloroform phase was removed, and the radioactivity was determined using a scintillation fluid consisting of 450 mg of 1,4-bis(2-[5-phenyloxazolyl]) benzene and 16.5 g of 2,5-diphenyloxazole dissolved in a mixture of 2 liters of toluene and 1 liter of Triton-X.

RESULTS

Growth of tms-12 and tms-12$^+$ at the permissive (34 C) and restrictive (48 C) temperatures. *tms-12$^+$* and *tms-12* grown at 34 C in minimal enriched medium consist of bacilli containing from one to three fully formed cross walls; that is, each bacillary CFU actually represents two to four individual, wall-bound cells. At this temperature, growth of the mutant and wild type was indistinguishable with respect to generation time and morphology.

Over a 2-hr incubation at 48 C, the rate of increase in cell mass, as measured by OD_{660nm}, was about the same in cultures of *tms-12$^+$* and *tms-12* (Fig. 1a). However, whereas the average length of individual cells in the *tms-12$^+$* population remained constant, cell length in the mutant population showed an exponential increase (Fig. 1b). At the same time, in the *tms-12* population, cross walls which had been previously formed at 34 C went on to separate during the first hr of exposure at 48 C; this resulted in a residual two- to threefold increase in bacillary CFU (Fig. 1c). By contrast, the total number of individual *tms-12* cells (as determined by bacillary CFU times average number of individual cells per bacillus) increased only 10% during the first 80 min at 48 C. That is, only 1 out of every 20 to 30 bacilli completed a "new" cross wall at 48 C. Mutant filaments examined after 60 min of 48 C incubation are 3.5 times the length of a normal individual cell; electron microscopy examination shows that the nuclei are apparently discrete and segregated along the length of the filament and that no septa are present in any stage of formation (Fig. 2).

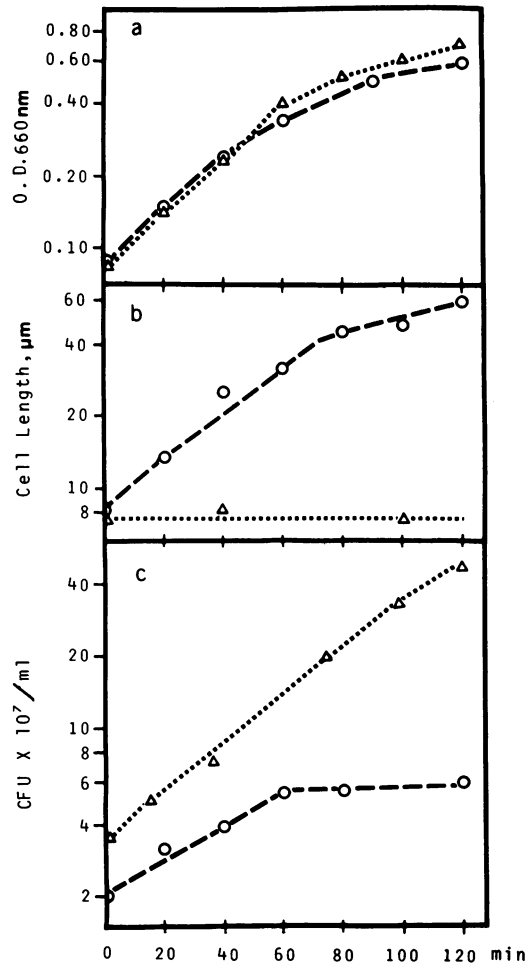


FIG. 1. *Growth of tms-12 and tms-12$^+$ at 48 C. Exponentially dividing populations of cells grown at 34 C in enriched minimal medium were exposed to 120 min of incubation at 48 C. During this period the growth was monitored in three ways. (a) The increase of culture density was followed by OD_{660nm}. (b) Microscopic measurements of the average individual cell length were made on wall-stained samples. Standard deviations for cell length were ±3.3 µm at 0 time, ±8.7 µm in the 60-min filaments, and ±14.9 µm in the 120-min filaments. (c) the number of viable bacillary colony-forming units was determined. Data from a typical experiment are shown. Symbols: tms-12$^+$, Δ; tms-12, O.*

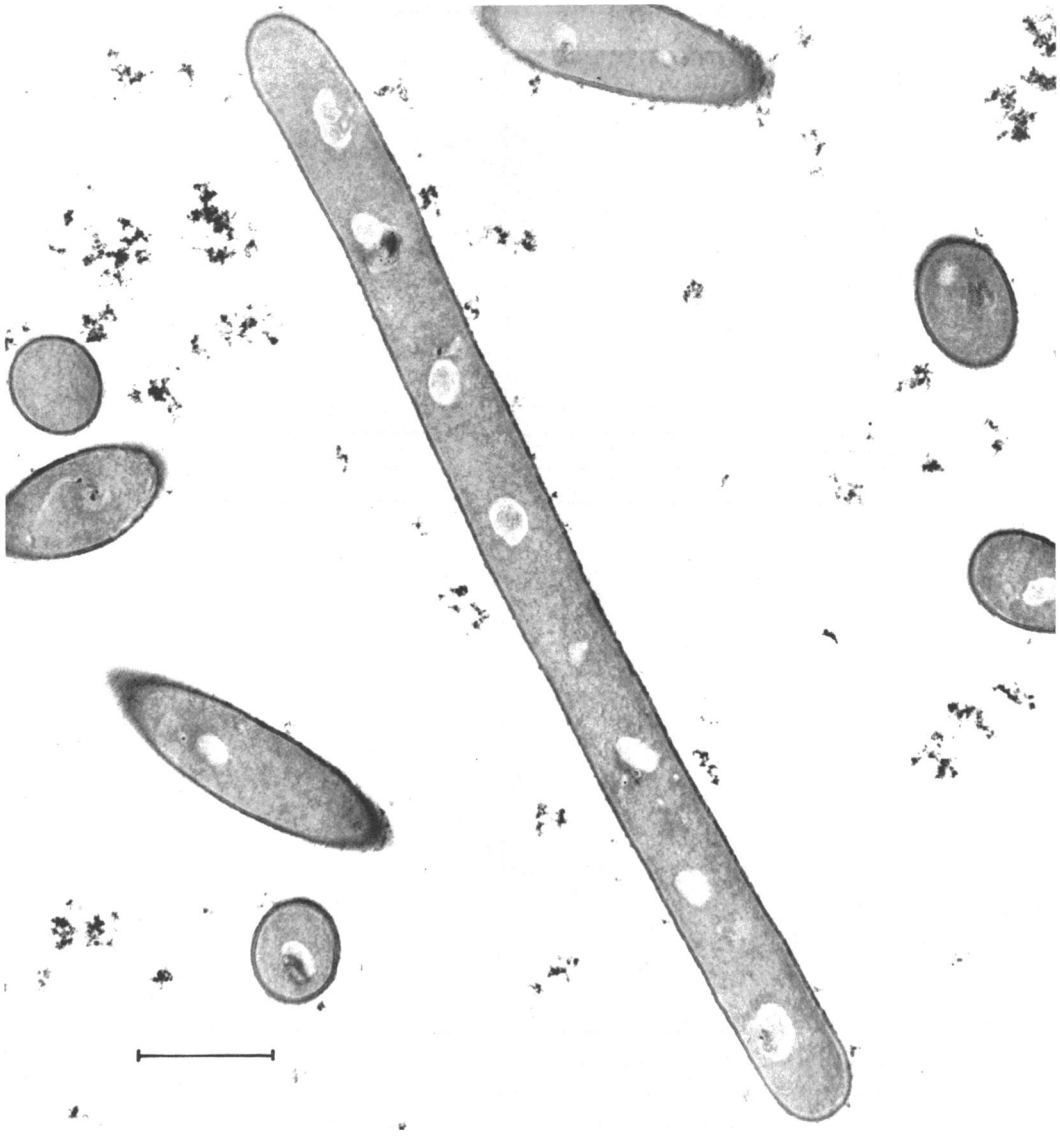
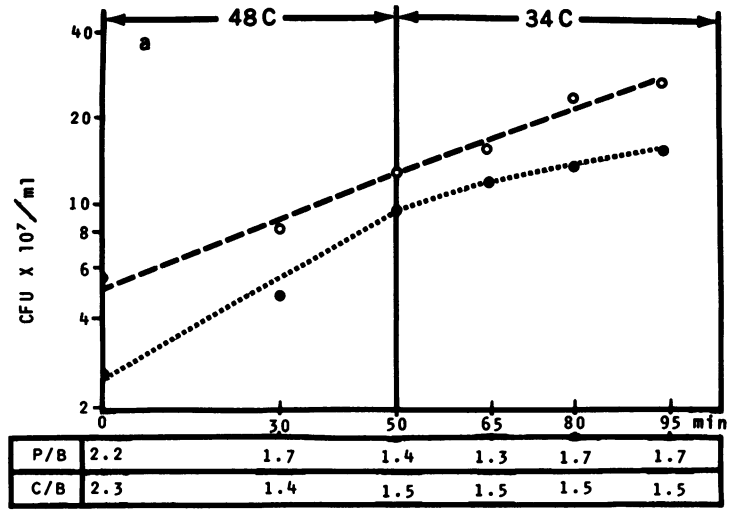
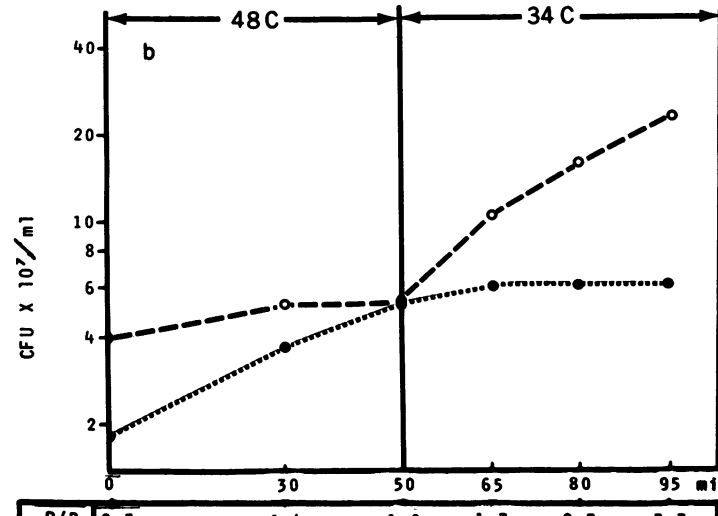


FIG. 2. *Electron micrograph of a typical filament of tms-12 formed after 60 min of incubation at 48 C. Samples were prepared for examination as described by E. Neale (Ph.D. dissertation, Georgetown University, 1969).* ×*23,400. Magnification marker 1 μm.*

Recovery of septation in filaments re-exposed to 34C. Filaments of *tms-12* formed during fairly short incubations at 48 C recover the ability to septate when returned to the permissive temperature. The occurrence of septation was monitored in *tms-12*$^+$ and *tms-12* populations both by determination of the number of viable protoplasts released per bacillus and by microscopic observation of cross walls (Fig. 3). Exponentially dividing populations grown at 34 C were incubated at 48 C for 50 min and then returned to 34 C for 45 min. For both *tms-12* and *tms-12*$^+$ the numerical ratios of individual cells to bacilli and of protoplasts to bacilli were similar, indicating that the survival of protoplasts under these conditions was close to 100%. Some septa form in mutant filaments during the first 15 min of re-exposure to 34 C. After 45 min the total number of protoplasts obtainable from filaments is equivalent to that from a parallel culture of *tms-12*$^+$. During 45 min of re-exposure to 34 C, there is no increase in the number of bacilli in the mutant population; however, after 60 min at 34



P/B	2.2	1.7	1.4	1.3	1.7	1.7
C/B	2.3	1.4	1.5	1.5	1.5	1.5



P/B	2.3	1.4	1.0	1.7	2.7	3.7
C/B	2.1	1.6	1.2	1.8	2.5	3.5

FIG. 3. *Septation in cultures of tms-12⁺ (a) and tms-12 (b) during a 50-min incubation at 48 C followed by a 45-min incubation at 34 C. Cells were grown in osmotically stabilized enriched minimal medium. Numbers of viable protoplasts (O) and bacilli (●) are shown. P/B ratio shows the average number of protoplasts obtained per bacillus for each time point. C/B ratio shows the average number of individual cells present per bacillus at each time point as determined by microscopic examination of wall-stained samples.*

C new bacillary CFU appear as the septa proceed to separation. Thus, filaments of *tms-12* recovering at 34 C require 60 min for the complete division cycle from septum initiation to daughter cell separation. After 120 min of re-exposure to 34 C the number of bacilli in the mutant population is restored to that of a parallel culture of *tms-12⁺* (data not shown).

Additional information on the re-appearance of septa in mutant filaments was obtained by microscopic determinations of changes in both the number of cross walls per filament and in the average length of individual cells during re-exposure to 34 C (Fig. 4). By this method, it is not possible to distinguish stages in cross wall formation; cross walls are presumed to be visible when they are at or near completion. No cross walls appear until after 10 min of incubation at 34 C; a burst of cross wall formation occurs between 15 and 20 min after transfer to

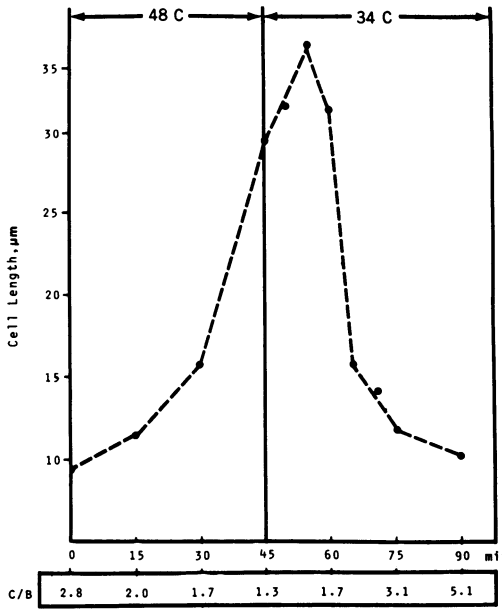


FIG. 4. *Microscopic determinations of the changes in the average individual cell length and in the ratio of individual cells to bacilli (C/B) accompanying incubation of tms-12 at 48 C and then at 34 C. Cells were grown in enriched minimal medium.*

34 C. After 45 min at 34 C the full complement of cross walls has formed and the normal average individual cell length has been restored. The first cross walls formed appear to have the thickness of the peripheral wall and are positioned at a distance of one normal cell length from the end of the filaments. By 45 min these cross walls are about twice as thick and additional cross walls have formed at normal intervals along the filament length. Occasionally two cross walls are seen to form so close to each other that they probably do not enclose any nuclear material.

Recovery of mutant filaments re-exposed to 34 C for short periods. To determine the length of re-exposure to 34 C necessary to permit septation to be re-instated in mutant filaments, *tms-12* cells were incubated at 48 C for 50 min, re-exposed to 34 C for 0, 5, 10, 15, and 25 min, and then returned to 48 C for a total incubation period of 75 min. After this time, the extent of septation was determined by microscopic examination (Table 1). Re-exposures of 10 or 15 min, but not of 5 min, permitted some septa to continue onto completion at 48 C. This is concluded from the increases in the ratios of individual cells to bacilli and the decreases in the average individual cell lengths. Comparing these results with the time sequence of uninterrupted septal recovery at 34 C (see Fig. 4), it can be seen that, whereas very few septa have formed after 10 min of 34 C re-exposure, such an incubation is sufficient to allow an increased complement of septa to be formed later at 48 C, a complement equivalent to that which would have appeared after a continuous 20 min of re-exposure to 34 C.

Effect of inhibition of DNA synthesis on septation. To determine if ongoing nuclear replication was necessary for septation or for recovery from the temperature-induced septation defect in *tms-12*, or both, *tms-12* and *tms-12*$^+$ cells were incubated at 48 C for 50 min and then exposed simultaneously to inhibitors of DNA synthesis and to 34 C for 45 min. 6-(*p*-Hydroxyphenyl-azo)-uracil (HPUr) and nalidixic acid were used at final concentrations of 80 µM and 15 µg/ml, respectively. These concentrations have been cited as being completely inhibitory to nuclear replication (9, 19) and were found here to be 90 to 95% effective in the suppression of ^{3}H-thymidine incorporation into cold 5% trichloroacetic acid precipitates (data not shown). At these concentrations, both inhibitors produced a 45 to 60% reduction in the growth of *tms-12*$^+$ cells as measured by increases in mass and bacillary CFU during the 45 min of re-exposure to 34 C. Under these conditions of inhibition of DNA synthesis, mutant filaments still formed one to 2 new septa during the recovery period at 34 C (Table 2).

When *tms-12*$^+$ and *tms-12* cells are given HPUr at the time of 48 C exposure, incubated at this temperature for 50 min, and then returned to 34 C for 45 min in the continued presence of this inhibitor, two effects on septation were noted (Table 3). First the individual cells of *tms-12*$^+$ were found to elongate, indicating that increases in cellular volume are continuing although DNA replication is curtailed, and that septation is delayed. Second,

TABLE 1. *Effect of brief re-exposures to 34 C on the septation of mutant filaments formed by 50 min of incubation at 48 C*

Duration of re-exposure to 34 C (min)	Duration of further incubation at 48 C (min)	Final ratio of individual cells per bacillus (C/B)[a]	Final average length of individual cells[a] (µm)
0	25	1.1	38.0
5	20	1.3	23.5
10	15	2.4	10.8
15	10	2.6	9.5
25	0	2.5	12.0

[a] Measurements represent the average of results obtained from three separate experiments.

TABLE 2. *Effect of inhibitors of DNA synthesis on the recovery of septation in mutant filaments formed during a 50-min exposure to 48 C*

Inhibitor[a]	Concn	Ratio of viable protoplasts to bacilli after 45 min at 34 C[b]	
		tms-12$^+$	*tms-12*
None		1.5	4.0
Nalidixic acid	15 μg/ml	1.2	2.5
HPUr	80 μM	1.6	2.7

[a] Inhibitors were present throughout recovery at 34 C.

[b] Ratios represent the average of determinations from three separate experiments. At the time of transfer to 34 C, the ratios for tms-12 were always close to 1.

TABLE 3. *Effect of HPUr on growth and septation of tms-12 and tms-12*$^+$ *at 48 C and during re-exposure to 34 C*

Strain	HPUr[a] (80 μM)	Successive incubations		Avg length of individual cells[b] (μm)	No. of dividual cells per bacillus (C/B)[b]
		48 C for 50 min	34 C for 45 min		
tms-12$^+$	−	+	−[c]	6.5	1.9
		+	+	8.1	1.3
	+	+	−	11.8	1.1
		+	+	16.5	1.1
tms-12	−	+	−	28.5	1.0
		+	+	9.3	5.0
	+	+	−	22.2	1.4
		+	+	22.2	2.6

[a] When present, HPUr was added at the time of exposure to 48 C and was present throughout the remaining incubations.

[b] Measurements represent the average of two separate experiments.

[c] Indicates that there was no 34 C exposure; measurements were taken directly following 48 C exposure.

septa form in mutant filaments during the recovery period to the same extent regardless of whether DNA synthesis was inhibited both during filamentation at 48 C and during recovery at 34 C, or only during recovery at 34 C (see also Table 2). In both cases septa partition off cells of greater than normal length which contain at least one nuclear region (as observed microscopically in wall-stained preparations).

Effect of inhibitors of RNA and protein synthesis on recovery of septation. Inhibitors of protein and RNA synthesis were added to filaments of *tms-12* at the time of transfer to 34 C and 7 min after transfer to determine if new protein or RNA synthesis was necessary for recovery of septation. Inhibitors were used at two concentrations, both of which were inhibitory to growth as measured by increases in mass. Only the higher concentrations were found to block completely the incorporation of ^{3}H-phenylalanine into cold 5% trichloroacetic acid precipitates (data not shown). Chloramphenicol (500 μg/ml), puromycin (500 μg/ml), and actinomycin-D (40–100 μg/ml) when present throughout the 45-min re-exposure to 34 C stopped septation almost completely; that is, the ratio of individual cells to bacilli remained low, and the average individual cell length was not decreased (Table 4). Similar concentrations of these inhibitors added after 7 min of 34 C recovery, that is, prior to the appearance of septa (see Fig. 4), and present during the remaining 38 min of recovery, did not interrupt the formation of septa. Lower concentrations of chloramphenicol (200 μg/ml) and of puromycin (100 μg/ml) scarcely inhibited septal recovery even when added at the beginning of re-exposure to 34 C.

In these experiments septation was assessed only by microscopic examination as these inhibitors were found to decrease the survival of protoplasts of both *tms-12* and *tms-12*$^+$. This decrease in the viability of protoplasts could be

TABLE 4. *Effect of inhibitors of RNA and of protein synthesis on the recovery of septation in mutant filaments formed during a 50-min incubation at 48 C*

Inhibitor	Concn (μg/ml)	Time when inhibitor was added following transfer to 34 C (min)	Characterization of cells following 45 min of re-exposure to 34 C	
			Avg length of individual cells[a] (μm)	No. of individual cells per bacillus[a] (C/B)
None			7.0	5.0
Chloramphenicol	200	0	12.3	2.3
	500	0	20.2	1.6
	500	7	11.1	3.1
Puromycin	100	0	8.2	3.1
	500	0	21.1	1.4
	500	7	13.4	2.4
Actinomycin D	40	0	17.0	1.6
	100	0	19.0	1.6
	100	7	10.2	3.3

[a] Measurements represent the average of three separate determinations. At the time of transfer to 34 C, the average individual cell length was 28 μm, and the number of individual cells per bacillus was 1.2.

accounted for by a decrease in the stability of the membrane which in turn might be due to an increase in the ratio of lipid to protein components, found to occur in the presence of inhibitors of protein synthesis (44).

Synthesis of mucopeptide and protein during growth at 48 C and recovery at 34 C. It was considered that filamentation might be associated with a decrease in the rate of synthesis of cell wall material in relation to that of other cellular components and that a relative accumulation of cell wall might occur during the septal partitioning of these filaments upon recovery (smaller cells have a larger surface to volume ratio). Cells were labeled with L-^{3}H-alanine during a 40-min exposure to 48 C and subsequently during 50 min of recovery at 34 C. Samples were removed at 10-min intervals and precipitated with cold 5% trichloroacetic acid, and the precipitates were fractionated according to the procedure of Park and Hancock (35). The accumulation of mucopeptide as compared with that of total cell protein was similar in wild type and mutant populations (data not shown).

Synthesis of membrane during growth at 48 C and recovery at 34 C. The defect in septation in *tms-12* cells exposed to 48 C could result from alterations in the synthesis or composition, or both, of the membrane. Accordingly, the accumulation of membrane in *tms-12* and *tms-12*$^+$ was compared during exposure to 48 C for 45 min and to 34 C for an additional 45 min. Membrane synthesis was monitored as the accumulation of ^{3}H-sodium acetate label into the chloroform-soluble or lipid fraction. In *B. subtilis* essentially all the cellular lipid is present in the membrane fraction (6).

The accumulation of ^{3}H-sodium acetate label into the lipid fractions showed an apparent decline upon exposure to 48 C in both *tms-12* and *tms-12*$^+$ (see Fig. 5). During extended incubation at 48 C, accumulation of lipid resumed in both populations but the lipid content of *tms-12* remained reduced relative to that of *tms-12*$^+$. When the cultures were reexposed to 34 C, the mutant population showed an increase in the accumulation of lipid relative to *tms-12*$^+$ so that after 25 min at 34 C the content of lipid in the two populations became equivalent. This accelerated lipid accumulation in the mutant occurs at the same time as recovery of septation in these filaments (see Fig. 5).

This alteration in lipid synthesis is apparently accompanied by an alteration in membrane protein synthesis also. Membranes isolated from mutant and wild-type populations grown at 48 C for 80 min or at 34 C to an equivalent density showed similar protein to lipid ratios (micrograms per micrograms) at each temperature. For the wild type this ratio was 4.1 at 34 C and 6.6 at 48 C, for the mutant, 3.5 and 5.2, respectively. Estimates of the lipid content of the membranes were made on the basis of phosphorus assays of their chloroform-methanol-soluble fraction. It was assumed that phosphorus constitutes 2.7% by weight of *B. subtilis* lipids (33).

In a further effort to pinpoint the mutational lesion of *tms-12*, a preliminary qualitative comparison was made between the membrane proteins and the phospholipids of strain *tms-12*$^+$ and those of strain *tms-12*. Membranes were isolated from both strains grown either at 48 C for 80 min or at 34 C to an equivalent density. The membranes were solubilized with SLS and their proteins separated on acrilamide gels containing 0.2% SLS (43). Following the methods of Inouye and Guthrie (22) for membrane solubilization and staining of the gels for protein, 27 bands could be clearly distinguished. The banding patterns obtained from membranes of *tms-12* and *tms-12*$^+$ grown at either 34 C or 48 C appeared identical (data not shown).

The lipids extracted from *tms-12* and *tms-12*$^+$ cells grown at 34 and 48 C, as above, were chromatographed on silica gel thin-layer plates in chloroform-methanol-acetic acid-water (100:50:14:6) as described by Bertsch et al. (5). Phospholipids were detected by means of a phosphorus stain (45); no qualitative differences were noted in these samples in the migration patterns of the four major phospholipids (data not shown).

Properties of the cell walls of tms-12 and tms-12$^+$ cells grown at 34 C and 48 C. Several properties of the cell walls of *tms-12* and *tms-12*$^+$ cells were compared after growth at both temperatures to determine if alterations in the composition of the walls accompanied the observed alterations in septation of the mutant at 48 C. Estimates of the amount of wall recovered per unit of cell mass indicated that both cell types grown at 48 C for 80 min possessed 1.5 to 2 times as much wall as when grown at 34 C to an equivalent density. The molar ratio of phosphorus to hexosamine was similar, 1.30 to 1.36, in wall samples isolated from both cell types after growth at 48 C for 80 min or after growth at 34 C to an equivalent density.

The ability of the wall to provide osmotic protection was compared in the various cul-

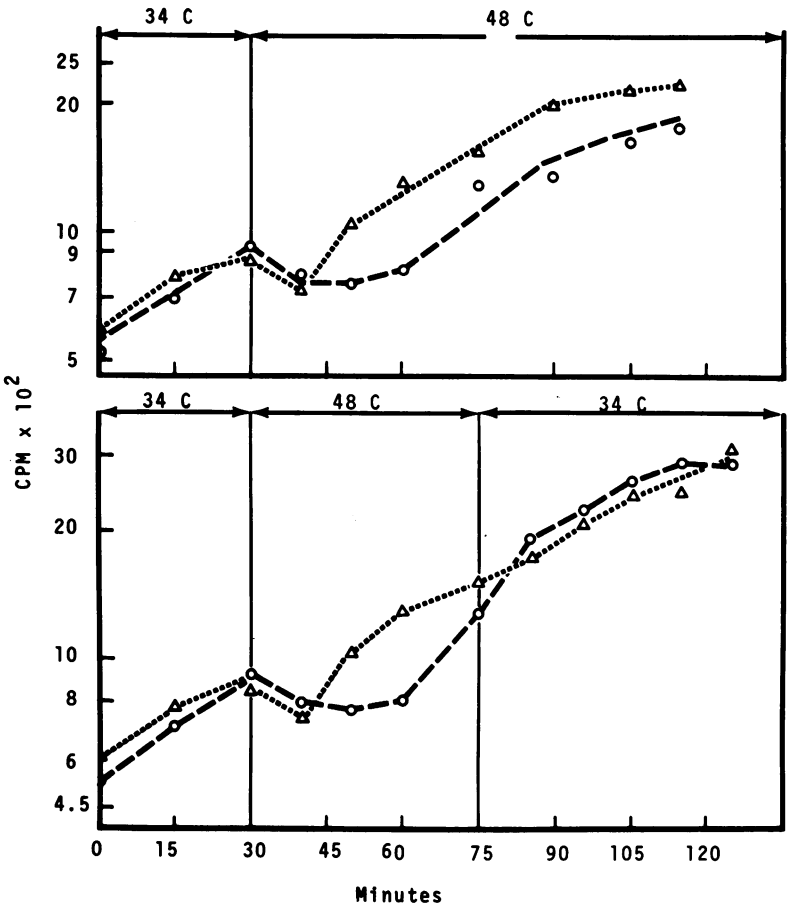


FIG. 5. *Synthesis of lipid at 34 and 48 C as determined by the incorporation of ^{3}H-sodium acetate label into the chloroform-soluble cell fraction. Cells were grown in enriched minimal medium. Each point represents the average of two separate experiments. Symbols: tms-12$^+$, Δ; tms-12, O.*

tures by measuring survival after osmotic stress. Following growth in osmotically stabilized enriched minimal medium, the cultures were diluted in a dilute salts solution and plated. All of the cultures, including mutant filaments formed after 80 min at 48 C, remained fully viable after this treatment.

The autolytic activity of isolated walls was also determined. Parallel cultures of *tms-12* and *tms-12*$^+$ were harvested after 40 and 80 min of growth at 48 C (OD_{660nm} of 0.25 and 0.60, respectively) and after growth at 34 C to equivalent densities. Walls isolated from both *tms-12*$^+$ and *tms-12*, when grown at 48 C for 80 min, showed a decreased rate of autolysis as compared to their activity when harvested after 40 min at this temperature or at 34 C (Table 5). In addition, the walls of mutant but not of wild-type cells showed an increase in autolytic activity after growth at 34 C to higher cell densities. No marked deviations from the above patterns were noted when walls were permitted to autolyze at 48 C rather than at 34 C, although the rates of autolysis at 48 C were two to three times faster than at 34 C (Table 5). When the wall samples were first heated to inactivate endogenous autolysin and then exposed either to lysates from unheated walls of *tms-12* or to 500 μg of lysozyme/ml, equal specific reaction rates of lysis were noted for all samples, 4.2×10^{-3} per min and 29.3×10^{-3} per min, respectively. This suggests that the observed differences in autolytic activities between strains *tms-12* and *tms-12*$^+$ are due to alterations in the activity of the autolysin(s), rather than to variations in the susceptibility of the mucopeptide substrates.

DISCUSSION

A temperature-sensitive mutant of *B. subtilis*, *tms-12*, has been used here to elucidate the interrelationship of septation with other

TABLE 5. *Rates of autolysis of walls isolated from cells grown at 34 or at 48 C*

Strain	Temp of growth[a]	OD_{600nm} of culture[a]	Specific reaction rates of hydrolysis at pH 9.6[b]	
			34 C	48 C
tms-12$^+$	34 C	0.25	6.3	15.5
		0.60	6.3	10.0
	48 C	0.25	7.1	21.5
		0.60	4.0	10.0
tms-12	34 C	0.25	9.3	23.2
		0.60	12.7	35.0
	48 C	0.25	9.5	19.2
		0.60	2.6	7.0

[a] Cells were grown in enriched minimal medium. Those harvested at an OD of 0.25 were grown at 48 C for 40 min; those harvested at an OD of 0.60 were grown at 48 C for 80 min.

[b] The specific reaction rate of hydrolysis (R) was calculated by use of the following formula: $R = \log C_o/C_x \times 10^{-3}/x$ min where C_o = the initial OD_{600nm} and C_x = the OD after x min of hydrolysis. Rates represent the average of three determinations on walls isolated in two separate experiments.

cellular processes and to dissect the series of events involved in septum formation. At the restrictive temperature, 48 C, this mutant is blocked in an early stage of septum formation, here called septum initiation, which occurs prior to the actual morphological appearance of the septum. This blockage leads to filamentation of the mutant at 48 C and is relieved upon re-exposure of the filaments to the permissive temperature, 34 C.

Several lines of evidence suggest that both septum assembly and subsequent separation of daughter bacilli can proceed in mutant cells at the restrictive temperature. When a population of mutant bacilli, each of which contains from two to four individual cells, is incubated at 48 C for 60 min, the number of viable bacilli increases about three fold, whereas the number of individual cells per bacillus (determined by microscopic examination) decreases to one as the cells filament. Moreover, the total number of individual cells present in the population increases by 10% during this period at 48 C. This 10% increment presumably stems from bacilli which have passed the phase of septum initiation in their cell cycle during the previous incubation at 34 C and can then continue subsequent steps in division at 48 C. Electron microscopy examination of filaments formed after 60 min of incubation at 48 C shows that in fact no septa are present in any stage of formation. Although this could also be explained by a resorbtion of partially formed septa during filamentation, the noted increment in individual cell numbers supports the conclusion that partially formed septa go on to completion at 48 C. This conclusion is further substantiated by the experiments in which filamented cells were exposed to 34 C for a period of time insufficient for the development of visible cross walls, but subsequent to which filaments could go on to form a limited number of cross walls at 48 C.

The recovery of filaments at 34 C permits further examination of the mutational defect and of the events of septation. The first completed cross walls begin to appear after the filaments have been re-exposed to 34 C for 10 min, and a burst of septum formation occurs after 15 to 20 min at 34 C. New bacilli do not begin to appear until after 60 min of recovery. Thus, there is a 45-min delay between the completion of septa and the subsequent separation of daughter bacilli. The recovery process is found to be dependent on the synthesis of RNA and protein only during the first 7 min of re-exposure to 34 C. Either recovery from the mutational defect or septum initiation, or both, requires these syntheses but subsequent steps in septation can proceed independently of them. Septal recovery proceeds when the synthesis of DNA is stopped through the use of inhibitors. This indicates that neither septum initiation nor septum assembly require ongoing DNA synthesis and suggests that, if DNA replication exerts control over septation, it does so prior to the time of expression of the mutational defect.

The mutational defect can be further examined by comparison with other causes of filamentation in bacteria. In particular, interference with nuclear replication or condensation, or alteration of the composition and synthesis of the cell wall can result in filamentation. Experiments in which the synthesis of DNA is curtailed in *Escherichia coli*, either through the use of mutants (1, 18, 21, 29) or with inhibitors of DNA synthesis (13, 27), have shown that septation does not normally proceed without prior nuclear replication. In *B. subtilis*, on the other hand, septa have been shown to form during extended inhibition of the synthesis of DNA, with the result that anucleate cells are formed (16). The observation made here that wild-type bacilli elongate in the presence of an inhibitor of DNA synthesis, however, suggests that in *B. subtilis*, also, there is a connection between nuclear replication and septation. However, the defect in septation of mutant *tms-12* does not

appear to be accompanied by any alterations in its nuclear replication. As noted by Copeland and Marmur (14) and confirmed by us (data not shown), there is no change in the rate of accumulation of DNA in *tms-12* cells as compared to *tms-12*$^+$ cells during a 2-hr incubation at 48 C. Moreover, as already noted, the mutant block to septation can be overcome at 34 C in the presence of inhibitors of DNA synthesis added either throughout the recovery period alone or throughout both filamentation and recovery. Recent studies on the effects of polyamines on nuclear condensation have suggested that some degree of condensation may be necessary for septation, so that septa do not form so as to impinge upon nuclear material (31). Electron microscope examination of *tms-12* filaments suggests that the nuclei are discrete and spaced at normal intervals throughout the length of the filaments (Fig. 2). Moreover, a comparison of the spermidine and putrescine content of *tms-12*$^+$ and *tms-12* cultures grown at 34 and 48 C did not reveal any differences which could account for the filamentation behavior of *tms-12* (E. J. Herbst, *personal communication*).

Alterations in the synthesis of cell wall have also been found to produce filamentation in bacteria (M. L. Higgins and G. D. Shockman, CRC Critical Rev. Microbiol., *in press*). In particular, antibiotics or conditions which interfere with mucopeptide synthesis often cause filamentation of gram-negative bacteria (25, 28, 42). Other studies suggest also that interference with the synthesis of another wall polymer, teichoic acid, can similarly disrupt septation (Higgins and Shockman, *in press*). In comparing walls of *tms-12* with *tms-12*$^+$, which had been isolated from cells grown at the restrictive and permissive temperatures, no change was found in the ratio of the two major wall polymers, mucopeptide and teichoic acid. Both strains produced more wall at 48 than at 34 C. Further, the walls of *tms-12* filaments grown at 48 C continue to provide normal osmotic protection, indicating that they possess adequate rigidity.

Another property of the cell wall which is apparently co-ordinated with septation and separation is that of localized autolysis (42, 44). Changes in the autolytic activity of *tms-12* walls, as compared to *tms-12*$^+$ walls, occur both after extended growth at 48 C and as the population density increases at 34 C. These changes, however, do not relate in a simple interpretable way to the filamentation behavior of the mutant. Indirect evidence also suggests that some changes in surface properties occur during filamentation. Mutant filaments tend to clump after more than 2 hr of growth at 48 C. Also, osmium tetroxide fixation of filaments results in the formation of an abnormally soft and light brown pellet, as compared to that formed by *tms-12*$^+$ cells after growth at 48 C (*unpublished observations*). Studies concerning the role of the cell wall in septal recovery will be described in a forthcoming paper.

Two other hypothetical causes of filamentation in bacteria might be responsible for the block to the initiation of septation noted in *tms-12*. Since bacilli of normal length should contain relatively more surface area per unit volume than would elongated bacilli, any decrease in the rate of synthesis of membrane or wall components, or both, could retard septation preferentially over lateral surface extension. The synthesis of mucopeptide was not found to decrease during filamentation. However, the rate of cellular lipid accumulation, an index of membrane synthesis, was diminished in populations of *tms-12* as compared to those of *tms-12*$^+$ during 48 C exposure. When mutant filaments were returned to 34 C, an increased accumulation of lipid and the reformation of septa occurred concomitantly. The apparent decrease of membrane synthesis at 48 C could either be the cause of or the result of an inhibition of septation. At present, a favored hypothesis is that the *tms-12* mutant is defective in the synthesis or insertion, or both, of specific proteins or lipids, or both, needed for septal initiation, as distinct from septum formation or lateral extension of the surface. Such a biochemical alteration might well be overlooked by the relatively gross methods of examination of membrane composition used here. Slight changes in the lipid or protein constituents of the membrane, or changes in a localized region of the membrane might be responsible, then, for the mutant defect.

The *tms-12* mutant of *B. subtilis* is different from other temperature-sensitive divisionless mutants of *E. coli* and *Salmonella typhimurium*. In *tms-12* cells some residual septation occurs at the restrictive temperature; this is not true of the BUG-6 mutant of *E. coli* (39), nor of the 4a mutant of *S. typhimurium* (2) and indicates that these latter mutants are probably blocked in septum formation rather than specifically in the initiation of septation, or that they are blocked in daughter cell separation, or both. The requirements for protein and RNA synthesis, but not for DNA synthesis during recovery distinguishes *tms-12* from either of the *E. coli* mutants described by

Nagai *et al.* (34). Moreover, in filaments of *tms-12*, inhibition of protein synthesis by chloramphenicol at 48 C does not stimulate septation (*unpublished data*) as it does in the case of the septationless mutant of *E. coli* described by Zussman et al. (Fed. Proc. **30**:1120, 1971).

ACKNOWLEDGMENTS

We thank G. B. Chapman for his electron microscopy examination of the mutant, E. J. Herbst for polyamine determinations, N. C. Brown for his gift of HPUr, and J. C. Copeland for supplying us with *tms-12*. We also thank Anthony V. Furano and Loretta Leive for their helpful criticisms of this manuscript. Support was provided to X.O.B. by National Defense Education Act Title IV and by the National Science Foundation through predoctoral fellowships and to O.E.L. by grants GB 7204 and GB 27607 from the National Science Foundation and by Public Health Service grant AI 05972 from the National Institute for Allergy and Infectious Diseases.

LITERATURE CITED

1. Adler, H. I., and A. A. Hardigree. 1965. Growth and division filamentous forms of *Escherichia coli*. J. Bacteriol. **90**:223-226.
2. Ahmed, N., and R. J. Rowbury. 1971. A temperature-sensitive cell division component in a mutant of *Salmonella typhimurium*. J. Gen. Microbiol. **67**:107-115.
3. Ames, B. N., and D. T. Dubin. 1960. Role of polyamines in neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235**:769-775.
4. Anraku, N., and O. E. Landman. 1968. Control of the synthesis of macromolecules during amino acid and thymine starvation in *Bacillus subtilis*. J. Bacteriol. **95**:1813-1827.
5. Bertsch, L. L., P. P. M. Bonsen, and A. J. Kornberg. 1969. Biochemical studies of bacterial sporulation and germination. XIV. Phospholipids in *Bacillus megaterium*. J. Bacteriol. **98**:75-81.
6. Bishop, D. G., L. Rutberg, and B. Samuelssen. 1967. The chemical composition of the cytoplasmic membrane of *Bacillus subtilis*. Eur. J. Biochem. **2**:448-453.
7. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911-917.
8. Boylan, C. W., and J. C. Ensign. 1968. Ratio of teichoic acid and peptidoglycan in cell walls of *Bacillus subtilis* following spore germination and during vegetative growth. J. Bacteriol. **96**:421-427.
9. Brown, N. C. 1970. 6-(p-hydroxyphenylazo)-uracil: a selective inhibitor of host deoxyribonucleic acid replication in phage-infected *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. **67**:1454-1461.
10. Chapman, G. B., and J. Hillier. 1953. Electron microscopy of ultra-thin sections of bacteria. I. Cellular division in *Bacillus cereus*. J. Bacteriol. **66**:362-373.
11. Chen, P. S., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. Anal. Chem. **28**:1756-1758.
12. Clark, D. J. 1968. Regulation of deoxyribonucleic acid replication and cell division in *Escherichia coli* B/r. J. Bacteriol. **96**:1214-1224.
13. Cohen, S. S., and H. D. Barner. 1954. Studies on unbalanced growth in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **40**:885-893.
14. 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.
15. Donachie, W. D. 1968. Relationship between cell size and time of initiation of DNA replication. Nature (London). **219**:1077-1079.
16. Donachie, W. D., D. T. K. Martin, and K. J. Begg. 1971. Independence of cell division and DNA replication in *Bacillus subtilis*. Nature (London). **231**:274-276.
17. Ellar, D. J., D. G. Lundgren, and R. A. Slepecky. 1967. Fine structure of *Bacillus megaterium* during synchronous growth. J. Bacteriol. **94**:1189-1205.
18. Gherardi, M., and N. Sicard. 1970. Induction of filament formation and thymineless death in *Escherichia coli* K12. J. Bacteriol. **102**:293-295.
19. Goss, W. A., W. H. Dietz, and T. M. Cook. 1964. A DNA polymerase inhibitor. J. Bacteriol. **88**:1112-1118.
20. Helmstetter, C. E. 1968. Regulation of chromosome replication and cell division, p. 15-36. *In* G. M. Padilla, G. L. Whitson, and I. L. Cameron (ed.), The cell cycle. Academic Press Inc., New York.
21. Hirota, Y., A. Ryter, and F. Jacob. 1968. Thermosensitive mutants of *Escherichia coli* affected in the processes of DNA synthesis and cellular division. Cold Spring Harbor Symp. **33**:677-693.
22. Inouye, M., and J. P. Guthrie. 1969. A mutation which changes a membrane protein of *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **64**:957-961.
23. Kaback, H. R. 1968. The role of phosphoenolpyruvate-phosphotransferase system in the transport of sugars by isolated membrane preparations of *Escherichia coli*. J. Biol. Chem. **243**:3711-3724.
24. Koch, A., and M. Schaechter. 1962. A model for the statistics of the cell division process. J. Gen. Microbiol. **29**:435-454.
25. Landman, O. E. 1968. Protoplasts, spheroplasts, and L-forms viewed as a genetic system, p. 319-322. *In* L. B. Guze (ed.), Microbial protoplasts, spheroplasts, and L-forms. Williams and Wilkins, Baltimore, Md.
26. Landman, O. E., and S. Halle. 1963. Enzymically and physically induced inheritance changes in *Bacillus subtilis*. J. Mol. Biol. **7**:721-738.
27. Lark, K. G. 1966. Regulation of chromosome replication and segregation in bacteria. Bacteriol. Rev. **30**:3-23.
28. Lederberg, J., and J. St. Clair. 1958. Protoplasts and L-type growth of *Escherichia coli*. J. Bacteriol. **75**:143-160.
29. Leighton, P. M. and W. B. Donachie. 1970. Deoxyribonucleic acid synthesis and cell division in a *lon*$^-$ mutant of *Escherichia coli*. J. Bacteriol. **102**:810-814.
30. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1961. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
31. Maas, W. K., Z. Leifer, and J. Poindexter. 1970. Studies with mutants blocked in the synthesis of polyamines. Ann. N.Y. Acad. Sci. **171**:957-967.
32. Marr, A. G., P. R. Painter, and E. H. Wilson. 1969. Growth and division of individual bacteria, p. 237-262. *In* 19th Symposium of Society of General Microbiology. Cambridge University Press, London.
33. Mindich, L. 1970. Membrane synthesis in *Bacillus subtilis*. II. Integration of membrane proteins in the absence of lipid synthesis. J. Mol. Biol. **49**:433-439.
34. Nagai, K., H. Kaneko, and G. Tamura. 1971. Thermosensitive mutant of *Escherichia coli* requiring new protein synthesis to recover cellular division ability. Biochem. Biophys. Res. Commun. **42**:669-675.
35. Park, J. T., and R. Hancock. 1960. A fractionation procedure for studies of the synthesis of cell-wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. J. Gen. Microbiol. **22**:249-258.
36. Patch, C. T., and O. E. Landman. 1971. A comparison of the biochemistry and rates of synthesis of mesosomal

and peripheral membranes in *Bacillus subtilis*. J. Bacteriol. **107**:345–357.
37. Paulton, R. J. L. 1970. Analysis of the multiseptate potential of *Bacillus subtilis*. J. Bacteriol. **104**:762–767.
38. Previc, E. P. 1970. Biochemical determination of bacterial morphology and the geometry of cell division. J. Theoret. Biol. **27**:471–497.
39. Reeve, J. N., D. J. Groves, and D. J. Clark. 1970. Regulation of cell division in *Escherichia coli*: Characterization of temperature-sensitive division mutants. J. Bacteriol. **104**:1052–1064.
40. Rogers, H. J. 1970. Bacterial growth and the cell envelope. Bacteriol. Rev. **34**:194–214.
41. Rondle, C. J. M., and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. Biochem. J. **61**:586–589.
42. Schwarz, V., A. Asmus, and H. Frank. 1969. Autolytic enzymes and cell division of *Escherichia coli*. J. Mol. Biol. **41**:419–429.
43. Shapiro, A. L., E. Vinuela, and J. V. Maizel. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrilamide gels. Biochem. Biophys. Res. Commun. **28**:815.
44. Shockman, G. D., H. M. Pooley, and J. S. Thompson. 1967. Autolytic enzyme system of *Streptococcus faecalis*. III. Localization of the autolysin at the sites of cell wall synthesis. J. Bacteriol. **94**:1525–1530.
45. Vaskovsky, V. E., and E. Y. Kostetsky. 1968. Modified spray for the detection of phospholipids in thin-layer chromatography. J. Lipid Res. **9**:369.
46. Young, F. E. 1966. Fractionation and partial characterization of the products of autolysis of cell walls of *Bacillus subtilis*. J. Bacteriol. **92**:839–846.