THERMOSENSITIVE CELL DIVISION SEPTATION AND SPORULATION SEPTATION IN A MUTANT OF BACILLUS MEGATERIUM

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ANTHONY DARE HITCHINS

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This is to certify that the

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Harold L. Sadoff
Major professor

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ABSTRACT

THERMOSENSITIVE CELL DIVISION SEPTATION AND SPORULATION SEPTATION IN A MUTANT OF *BACILLUS MEGATERIUM*

By

Anthony Dare Hitchins

The objectives of this study were to isolate conditional cell division mutants of *Bacillus megaterium* ATCC 19213 and to use them to test the hypothesis that the early stages of bacterial spore formation are analogous to stages of normal cell division. Isolation of a thermosensitive mutant, TH 14, defective in cell division septation made it possible to test a specific corollary of the hypothesis: that sporulation septation and cell division septation are analogous processes.

Mutant TH 14 was isolated by replica plating colonies formed by survivors of N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis and of a subsequent D-cycloserine selection step at the restrictive temperature (37 C or higher). Premissively grown colonies of survivors were replicated to the restrictive temperature and poorly grown replicate colonies were picked for subculture. TH 14

was chosen from such isolates because it formed filaments at the restrictive temperature. The filaments were shown to be aseptate and multinucleate by electron microscopy of ultrathin sections and Giemsa staining. Filaments were formed in nutrient broth or in simple defined medium. In the latter, filamentation was independent of the carbon-source used for growth.

The sporulation frequencies of the mutant at the permissive temperature (30 C) and at the restrictive temperature (39 C) were 0.6 and 10^{-5}, respectively, whereas the corresponding values for the parent strain were 0.8 and 0.1. Revertants of TH 14, selected for ability to divide normally at restrictive temperature, concomitantly recovered the ability to sporulate. These facts plus the fact that the asporogenic property was not directly selected for in the original isolation of TH 14 suggest that the filamentation and asporogenic properties are due to the same mutation.

Asporogeny was due to a block in sporulation prior to septation (stage II) since no septation or later stages were detected in ultra-thin sections of filaments by electron microscopy. The deoxyribonucleic acid (DNA) yields of permissive and restrictive mutant cultures were equal. This suggests that the last round of DNA replication necessary for sporulation is not blocked. Stationary growth phase filaments shifted from restrictive to permissive conditions sporulated with a frequency of about 10^{-2}.

Spores were confined to the shorter filaments. It follows that short filaments at least are not inherently unable to sporulate due to some factor unrelated to their aseptateness. Sporulation occurred at higher frequency at the restrictive temperature if permissively-grown mutant cells were shifted-up before the last 1 to 2 rounds of DNA replication in the culture. Similarly, when mutant cells were shifted to the restrictive temperature about two cell divisions occurred before the decreased rate of growth and filamentation started. Together these results suggest that after shift-up two division septations or one division septation followed by one sporulation septation can occur before the effect of the mutation is detectable. In toto, the sporogenesis studies are consistent with the view that the TH 14 mutation affects both cell division septation and sporulation septation. This supports the hypothesis that the early stages (I and II) of sporulation represent a modified cell division.

The nature of the TH 14 gene product and its relationship to the cell division and sporulation septation processes is not yet known. However, the mutant TH 14 was affected in several noteable properties. It had a doubled mean generation time, as measured turbidimetrically or by DNA synthesis, in restrictive culture compared with that of the parent strain. The restrictively-grown mutant did not show any qualitative compositional changes in membrane proteins which are extractable in hot sodium dodecyl

sulfate plus 2-mercaptoethanol. However, the mutant had a reduced content of a small molecular weight protein(s), equivalent in size to cytochrome c, at the restrictive temperature. Also a membrane protein (molecular weight about 80,000) was partially derepressed in the restrictively-grown mutant. Additionally, unidentified cytoplasmic inclusions (diameter about 100 nm) of a nonmembranous nature, were found in the mutant at the restrictive temperature.

Filamentous growth of the mutant was not so sensitive to penicillin as was growth in the rod form. Growth in either form was equally sensitive to D-cycloserine or rifampicin at the concentrations tested.

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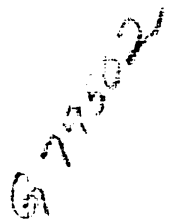

DEDICATION

To my wife and to my mother

ACKNOWLEDGMENTS

My sincere thanks for the guidance of my academic advisor Dr. Harold L. Sadoff throughout all aspects of my graduate program. Without his encouragement and financial support I would not have been able to carry out the program.

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GENERAL INTRODUCTION

Morphological differentiation is a common characteristic of living organisms. In multicellular organisms it is seen as the development of form (embryogenesis) or as a change of form (metamorphosis). The reverse process, dedifferentiation, may occur, sometimes with disastrous effect as in cancer. In unicellular organisms, morphological differentiation involves changes in shape and structure of single cells.

In the last 15 years, studies of differentiation in the simplest unicellular organisms, the procaryotes, and their viruses, have led to an increased understanding of the genetic and biochemical basis of differentiation. Aside from the intrinsic interest of procaryotic differentiation, the motivating force of these investigations has been the hope that they would provide realistic models for understanding differentiation in the more complex eucaryotic cells, especially those of multicellular organisms. In recent years, the relative success of this approach has prompted its extension to the unicellular eucaryotic organisms such as yeast. Despite the increase in knowledge about procaryotic differentiation, understanding of its initiation and control is still primitive.

Thus research into procaryotic differentiation remains of fundamental importance.

Bacterial spore formation (sporogenesis) is the most studied procaryotic differentiation process. It has been resolved into seven morphological stages and many of the chemical changes involved have been analyzed in detail. However, even such a simple differentiation system contains about two thousand different proteins as well as other complex polymers. The immense problem of auditing the compositional differences and similarities between the vegetative and spore forms has understandably led to focusing of attention on the differences rather than on the similarities. This seems to have led to the implication that understanding control of the synthesis of the differentiating molecules would be sufficient for understanding sporogenesis. Recently the validity of this viewpoint has been questioned so that interest has shifted to the way in which vegetative functions and components are adapted to the process of sporulation.

The characteristic function of the vegetative cell is the ability to replicate itself by the processes of growth and cell division. Interestingly, the early stages of sporulation resemble the process of cell division. Historically, this has been recognized ever since the advent of the use of the electron microscope in the late nineteen-fifties in studying sporulation. Many researchers

have commented on this similarity but without: (1) evaluating the evidence for the idea, (2) pointing out the importance of the differences from normal division to the later stages of sporulation, and (3) considering that the induction of sporulation may involve the way in which normal division is modified. In contrast, other researchers, by emphasis of the differences between normal division and sporulation, seem to imply that cell division and sporulation are unrelated.

As a result of this situation it was decided to test the hypothesis that the early stages of sporulation represent a modified cell division. This hypothesis is worth testing because it may clarify the nature of the transition from cell division to cell differentiation and vice versa that occurs in all differentiating or dedifferentiating cells.

The test involved isolating and studying the sporulation properties of a mutant defective in one of the stages of cell division: initiation of deoxyribonucleic acid (DNA) synthesis, DNA replication, segregation of the replicated DNA molecules, and cell division septation. Obviously, such mutations must be conditional since they affect the process that determines viability.

In fact, a thermosensitive filamentation mutant defective in cell division septation was used to test whether division septation and sporulation septation are

related. The results are described in this thesis. The thesis is divided into four parts: (1) a survey of the literature pertinent to the modified cell division hypothesis, (2) a description of the isolation, growth and sporulation properties of the thermosensitive mutant defective in cell division septation, presented in the form of a reprint of a symposium article, (3) a manuscript of a proposed publication, describing the membrane proteins and other properties of the mutant, and (4) a short summary.

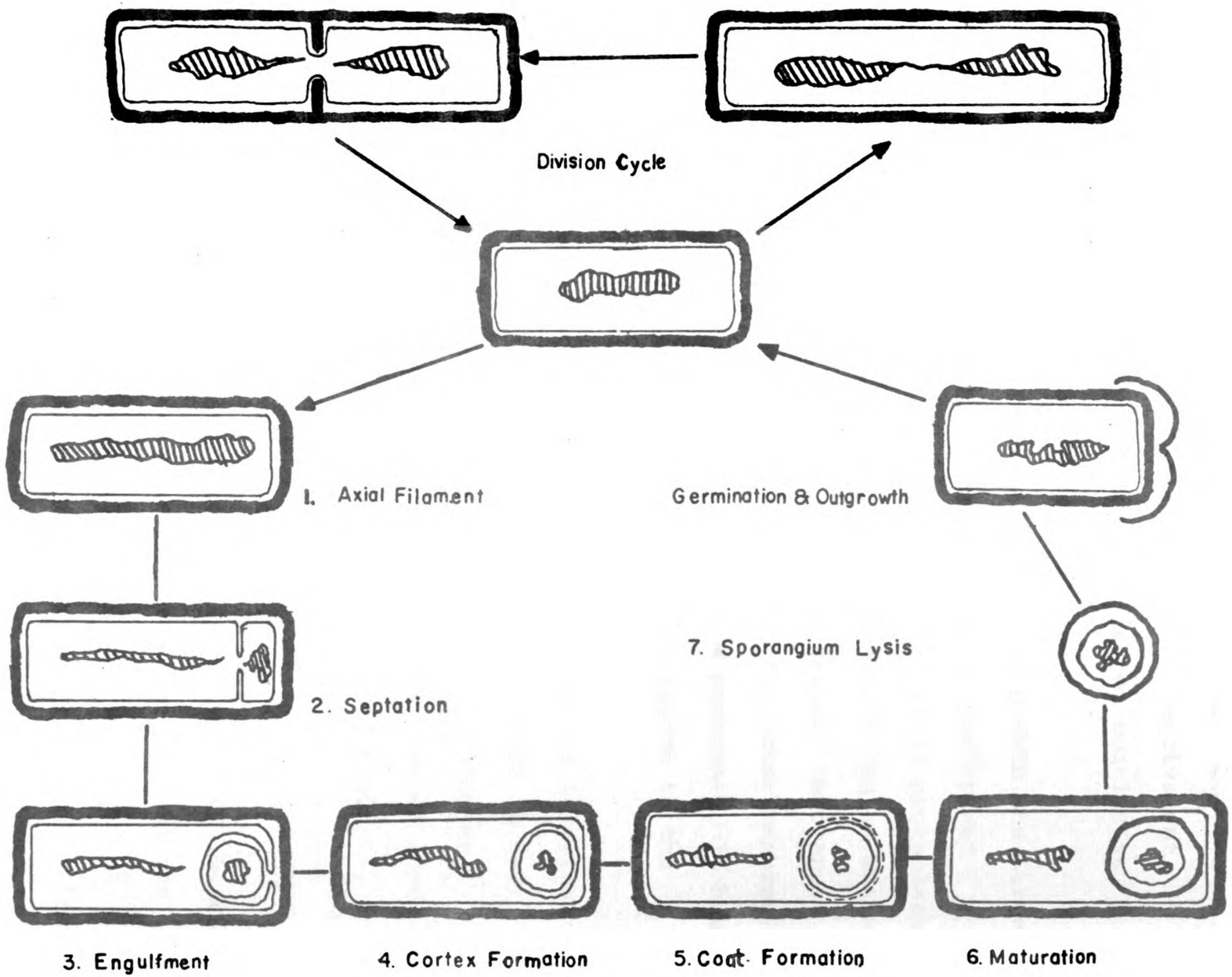
LITERATURE SURVEY

Morphology of Sporulation (Stages I-VII)

Bacterial spores are special cells that are resistant and dormant. More correctly, the spores are called endospores because they are formed inside a cell. A spore-containing cell is called a sporangium. Spore formation is confined mainly to rod-shaped cells of the family Bacillaceae, though a spheroidal-shaped organism, the coccus Sporosarcina ureae also forms spores. Recently certain filamentous actinomyces have also been shown to form endospores (12, 50). Sporulation occurs with high frequency at the end of growth in batch cultures although it does occur at lower frequency also during growth (85). However, in continuous cultures sporulation can occur with high frequency at low growth rates (15). Thus total starvation is unnecessary for sporulation to occur.

Seven morphological steps occur during spore formation (3, 26, 27, 64). These are diagrammatized in Figure 1. In stage I, the deoxyribonucleic acid (DNA) completes replication and becomes aligned on the long axis of the future sporangium; this is the axial filament or preseptation stage. Next, in stage II, the septation stage, a septum or partition is formed near one pole of

FIGURE 1.--Division cycle and stages of sporulation of a typical spore forming bacillus. The rod shaped cells are illustrated diagrammatically as they would appear in longitudinal and/or equatorial thin sections. Symbols: thick lines, cell walls; thin lines, membranes; dashed line or medium thickness line, spore coat; hatched areas, deoxyribonucleic acid (nucleoids). Cytoplasmic areas and the spore cortex zone have been left blank for clarity. Mesosomes and the exosporium have been omitted for simplicity. Scale: the width of a typical bacillus is about 1000 nm.


Division Cycle
1. Axial Filament
Germination & Outgrowth
2. Septation
7. Sporangium Lysis
3. Engulfment
4. Cortex Formation
5. Coat Formation
6. Maturation

the rod-shaped sporangium. This divides the sporangium into a large compartment and small compartment. These compartments can be regarded as protoplasts. Each compartment probably contains one genomic equivalent of DNA though the evidence for this is not conclusive (64, 84).

By means of cytoplasmic membrane growth the large protoplast engulfs or envelopes the small protoplast (Stage II, engulfment). As a result the small protoplast, ends up inside the large protoplast and also has an extra membrane around it called the outer forespore membrane. The ensemble is called the forespore. The outer membrane is derived from the larger protoplast's membrane. It has the novel feature of being inside out relative to the other membranes in the sporangium.

During stage IV, cell wall material is deposited between the outer and inner forespore membranes in the form of two concentric spheroidal shells. The outer thicker shell is called the cortex and the thinner inner one is called the germ cell wall or cortical membrane. The small protoplast and its primordial cell wall form the germ cell of the spore.

A coat made of several layers is deposited around the forespore in stage V (coat formation). Some of the sporangial cytoplasm is incorporated into the spore at this stage (64). Stage VI or maturation is an ill-defined

stage covering the period between coat formation (stage V) and stage VII, when the mature spore is released into the environment due to lysis of the sporangium. Biophysical changes such as increase in resistance to heat and radiation and increase in refractility are associated with stages IV and V.

The typical spore thus consists of the germ cell encased in cortical and coat layers. The final fate of the outer-forespore membrane is not clear. When conditions become suitable, the free mature spore germinates and the germ cell grows out to form a new vegetative cell.

A variety of morphological mutants have been isolated. These are characterized by blocks in development at specific stages of sporulation. Freese (27) has proposed the name cacogenic for developmental mutations causing abnormal forms. The normal genome would then be called protogenic.

Sporulation Biochemistry

Associated with the 6-8 hour long sequence of morphological changes occurring during sporogenesis are biochemical events involving metabolic shifts, the appearance of new metabolic products and modification of some vegetative components (45). Metabolic shifts occur early in sporulation because of carbon or nitrogen limitation due to exhaustion of the supply during growth. Derepression of enzymes occurs. Reserve material such as poly-β-hydroxybutyric

acid and by-products of growth such as acetate and pyruvate, are utilized for energy and carbon sources. This involves increased respiration, increased activity of the tricarboxylic acid (TCA) cycle with concurrent changes in enzyme patterns and in pH if the environment is only weakly buffered. Limiting energy, carbon or nitrogen sources are replenished by turnover of proteins and ribonucleic acid (RNA). DNA synthesis stops during stage I, the axial filament stage.

Many enzymes and biochemical products appear at specific times during sporulation. Exoprotease(s), antibiotic(s) and toxin(s) are excreted early in sporulation (84). Dipicolinic acid (DPA) accumulates in spores about stage IV. Associated with DPA synthesis is cortical peptidoglycan synthesis and calcium accumulation. Disulfide-rich coat protein(s) accumulate during stage V. In some species, there are spore associated structures such as exosporia and parasporal bodies which appear around mid-sporulation.

Some enzymes are modified post-translationally by proteolytic action, e.g. aldolase (79) and RNA polymerase (53). With RNA polymerase such structural modification (54) alters its transcriptional specificity with regard to the genome of phage ϕe (55). The possible relationship of protease, antibiotic(s) and enzyme modification to the control of sporulation has been discussed (78). At the

translational level changes in transfer-RNAs (107) occur which could conceivably have important consequences for sporulation morphogenesis. Changes in other nucleic acids appear to be relatively minor and have been reviewed elsewhere (17).

The sporulation specificity of the biochemical events is not always clear (30). Many of the metabolic changes could be typical of stationary phase metabolism in asporogenic bacteria. Other changes appear to be sporulation specific such as coat protein or DPA production. However, the fact that it is possible to obtain cacogenic forms such as DPA-less spores, coat-less spores or 'spore-less' coat protein suggests that production of even these substances need not be obligately coordinated with the whole process of sporogenesis. The situation resembles that observed with production of bacteriophage parts (22).

Early Sporulation (Stages I-II) as a Modified Cell Division

In this section, bacterial cell division (binary fission) is briefly outlined, and then the relationship of sporulation to cell division is introduced. Reviews covering the various aspects of cell division were consulted as indicated: DNA replication (5, 28), wall growth and wall septation (32, 101) and cytoplasmic membrane growth and septation (80).

Like sporulation, cell division has been resolved into a sequence of cytological stages at which mutational blocks may occur. These stages in order of occurrence are: initiation of DNA synthesis, DNA synthesis, segregation of duplicate genomes, septum formation by wall and membrane, and separation of the two resultant cells. Segregation of the DNA is semi-conservative.

The cell division septum is a partition formed across the middle of the cell at right angles to the direction of growth. It consists of cell wall material sandwiched between two cytoplasmic membranes. The wall layer of the septum is called the cross wall to distinguish it from side or lateral wall. The cross wall separates into two halves thus allowing cell separation. Presumably, the two halves were formed by the neighboring cytoplasmic membranes. After cell separation, the mature septum forms the new pole of the cell. The old pole is derived from the septum of the preceding cell division.

Separation is the last stage of cell division. Delay of separation results in chains of cells being formed. Due to this, completion of a septum is a more convenient way of defining the end of cell division. Thus physiological separation as judged by survival from phage infection or ultrasonication provides an endpoint before separation (11).

A major control of normal cell division in Escherichia coli involves the fact that septation can occur after completion of a round of DNA replication. However, initiation of new rounds can occur before an old one is completed. This control mechanism ensures proper segregation of genomes while allowing increased rates of DNA synthesis in rich medium. However, such a control is not always operative in wild type Bacillus subtilis (20) or in certain mutants of E. coli (35).

In contrast to sporulation, very little is known about the biochemical changes that occur throughout the cell cycle. Changes in phospholipid composition during septation (4) and a transient increase in glycerol incorporation at septation (13) have been reported.

Robinow (71) was the first investigator to point out the resemblance between the early stages of sporulation and cell division. The similarity was noted by successive authors in later years. Hitchins and Slepecky (34) reexamined the idea in 1969 and used it as a framework on which to arrange much of what was known about sporulation at that time. They concluded the idea was a viable one and proposed the following hypothesis: that the early stages (I and II) of sporulation are equivalent to a modified cell division and that this modification results from shift-down growth conditions. A copy of this review is appended to this thesis (see Appendix) because it is very pertinent to this literature survey.

In the following three sections, the reinterpretation of the first two stages of sporulation as modified stages of cell division and the consequences of the modification for later stages are discussed with special reference to relevant experimental findings obtained since 1969.

Reinterpretation of the Axial Filament (Stage I)

Although the axial filament configuration of the DNA in stage I has been considered a peculiar structure unique to sporulating cells there are reports of its occurrence in normal cell division of sporeformers (75, 84) and also in a non-sporeformer, E. coli (43). Thus, it has been suggested that axial filaments may be slowly segregating newly replicated DNA molecules. Consistent with this viewpoint is the continued synthesis of DNA during stage I (98) which presumably is equivalent to the last round of DNA replication since the spore germ cell contains half of the DNA originally present in the sporangium (45, 64). According to the modified cell division hypothesis, stage I can be regarded as completion of the DNA replication stage and the beginning of the segregation stage of cell division.

Recently Mandelstam et al. (14, 57) have reported interesting results obtained with the temperature-sensitive (ts)- 134 B. subtilis mutant of Mendelson and Gross (61).

Initiation of DNA synthesis is ts in this mutant. Cells transferred to 45 C complete a round of replication and can sporulate when transferred to sporulation medium at 35 C. However, the nature of the medium used at 45 C to complete the round is important. If the medium at 45 C is rich in amino acids, no sporulation occurs in sporulation medium at 35 C. If the 45 C medium was a poor one then sporulation was possible. The nature of the medium affected the disposition of the DNA molecules formed. In poor medium an axial filament was formed. In rich medium there were discrete nucleoids. This result seems to support the role of shift-down conditions in modifying cell division for sporulation suggested by Slepecky (91) and by Hitchins and Slepecky (34). Mandelstam et al. (57) concluded that sporulation is induceable only during a round of replication and not at the end of a round. Other experiments demonstrated that DNA synthesis was essential for sporulation to occur. Thus, as in normal cell division, a sporulation septum is not formed unless the preceding round of DNA replication is both initiated and completed.

Until recently it has been believed that the functioning of the TCA cycle is essential for sporulation of Bacillus species since certain asporogenous mutants have defective TCA cycles. Yousten and Hanson (116) studied an aconitase-less mutant that is blocked at stage 0 or I of sporulation with little or no axial

filament formation. They suggested two alternative explanations. The first was that the modified cell division hypothesis is not correct since the mutation did not affect normal cell division, though of course it would if the carbon source was acetate. Alternatively, they suggested that the round of DNA replication preceding sporulation is more sensitive than normal replication to adverse conditions since few or no axial filaments were produced. The second explanation would appear to be very likely in view of the results of Mandelstam, Sterlini and Kay (57). Presumably the TCA cycle block prevents a shift-down of metabolism thus precluding formation of the axial filament configuration of DNA and hence sporulation. It is not clear from the results of Yousten and Hanson (116) whether or not the last round of DNA replication did occur in the TCA cycle mutant. In any case, recently Carls and Hanson have found that certain TCA cycle mutants exhibit a sporulation frequence of 1 to 10% (6). Thus the idea that a functional TCA cycle is essential for sporulation is less clear than it used to be.

Studies with a conditional serine protease mutant (53) have led Santo et al. (82) to the conclusion " . . . that forespore formation may not be the result of an essentially vegetative asymmetric cell division. . . ." They reason that since sporulation genes are only expressed in sporulation due to the proteolytic modification of RNA

polymerase such genes cannot be involved in normal cell division. Thus the early stages of sporulation are not analogous to cell division. This does not prove that the early stages are not a modified cell division.

Since the completion of DNA synthesis and axial filament formation that occur during stage I are consistent with the modified cell division hypothesis, what about the segregation of the resulting daughter DNA molecules? Kogoma and Yanagita (44) showed that the segregation of the "old" and the "new" daughter DNA molecules into the spore germ cell is randomly determined in B. cereus. This agrees with the random segregation of daughter DNA molecules in B. subtilis cell division (77). However, the latter report conflicts with a previous one (21). Furthermore, determination of DNA segregation patterns during sporulation may be complicated by the demonstration of DNA excretion (about 40%) during B. subtilis sporulation (2). Balassa has recently reviewed the subjects of DNA synthesis during sporulation and the origin of spore DNA in greater detail than is possible here (3).

Septation (Stage II) as a Modified Division Septation

Septation, stage II of sporulation, involves partitioning of the future sporangium into two compartments by a process of membrane invagination due to its growth. Dividing a cell into two compartments is also the function

of the cell division septum. The common compartmentalization function of the two kinds of septa is the main reason for suggesting that division and sporulation septation are analogous processes. An additional reason is that the two kinds of septation have in common the property of completely segregating the daughter DNA molecules formed by the previous round of replication.

However, there are two very important differences between the two kinds of septa that have to be accounted for if their analogous nature is to be accepted. The first difference is the asymmetric location of the sporulation septum as opposed to the symmetric location of the cell division septum. The sporulation septum is much nearer to one of the poles of the rod-shaped future sporangium. This results in two unequal sized compartments. In contrast, the cell division septum is centrally located and septation results in two equal sized compartments (58). The second difference is that sporulation septa contain no detectable cell wall materials such as peptidoglycan whereas cell division septa do contain such material. The importance of these differences for subsequent events in sporulation will be discussed in the next section.

One reason for the asymmetry of the location of the sporulation septum could be that it is a novel process entirely unrelated to cell division septation. Indeed, Kretschmer (46) has proposed that the sporulation septum

may be the product of a reinitiated, additional membrane synthesis at an annular zone. This zone occurring at the newly formed poles of daughter cells would be normally responsible for cell elongation. The activity of this zone is usually repressed by the next division and remains repressed under starvation conditions. This alternative model for sporulation septation seems to be more applicable to stage III (engulfment) which involves post-septation membrane growth. Interestingly, even Kretschmer suggests that sporulation septation timing is related to initiation of cell division.

However, there is no a priori reason to assume that an asymmetrically located septum could not be related to a cell division septum. For instance, asymmetric septation is not specific to sporulation but occurs in a mutant of E. coli that forms DNA-less minicells (1), and in redivision of filaments (19). Thus asymmetry seems to be a cell division septation related phenomena but its cause is unknown.

In view of the postulated effect of shift-down growth conditions (34, 91) during stage I of sporulation (7, 105) and the implied support for this view from the work of Mandelstam and coworkers (14, 57) it seems likely that the unbalanced growth effect could continue into Stage II and result in asymmetric septation. The relative independence of sporulation septation and cell elongation is also sometimes seen with cell division and growth (16).

This effect of the medium in determining the choice between division septation or sporulation septation is well exemplified by the phenomenon of microcycle sporulation (106). Microcycle sporulation occurs when a cell, growing out from a germinated spore, forms a spore instead of dividing. This effect is caused by low levels of nutrients often resembling shift-down growth conditions. Similarly log-phase sporulation (85), the production of spores at low frequency under growth conditions emphasizes the alternative types of septation. The frequency of log phase sporulation varies with the nature of the carbon source. The choice between cell division septation and sporulation septation, i.e. between division and spore formation, can be regarded as a probability event that depends on environmental conditions (85).

The sporulation septa are not always peptidoglycan-less. Under certain conditions they contain cell wall material as when growth of stage II cells is rejeuvenated by addition of fresh nutrients (25). Also certain sporulation mutants have septa that contain cell wall material (69, 75, 76, 115). Similarly spontaneous aberrant forms which look like minicells, appear during sporulation of Clostridium sp. and these septa contain cell wall material (81). Spikes of wall material are associated with sporulation septa in B. subtilis (27, 114). Such observations suggest that under certain conditions

sporulation septa can be converted into division-like septa by addition of wall material.

Even though sporulation septa of B. cereus do not contain detectable cell wall material such material has been detected in 3 Clostridium species (109). Also there is evidence of changes in peptidoglycan being associated with septation. The situation is complex because the evidence is conflicting. Vinter (104) first reported a peak of new peptidoglycan synthesis associated with stage II in B. cereus and this has been confirmed in B. megaterium (R. Greene and R. A. Slepecky, Unpublished results; 34). However, Pitel and Gilvarg (66, 67) could not detect net synthesis of peptidoglycan in a diaminopimelic acid (DAP) requiring mutant of B. megaterium. Neither could the latter authors detect turnover at stage II. Even data for peptidoglycan turnover in vegetative cells is conflicting (32). Thus turnover is reported in B. subtilis (60) and in B. megaterium (8), but with their DAP-requiring B. megaterium mutant, Pitel and Gilvarg (66) found no turnover.

Chow and Takshashi (10) have recently studied the acid-soluble nucleotides of asporogenous mutants of B. subtilis. In the wild type strain the nucleotides, uridine diphosphate (UDP) galactose and UDP-N-acetyl-glucosamine increase at stage II. In a mutant blocked at stage II just after septum formation, these nucleotides also increased.

However, in a mutant blocked at stage 0, i.e. prior to axial filament formation there was no increase in these two nucleotides but a third one, UDP-glucose accumulated. These observations suggest that some changes in wall metabolism occur between stage 0 and stage II in B. subtilis. Consistent with this viewpoint is the observation by Hitchins and Slepecky that penicillin added at stage I prevents subsequent septation (33). Furthermore, binding studies with ^{14}C-benzylpenicillin (51) show a peak of binding presumably to glycopeptide transpeptidase associated with stage II. Binding studies with sproulation mutants confirmed this conclusion (73). However, penicillin also binds to the D-alanine carboxypeptidase of B. subtilis though it is postulated this enzyme may be an uncoupled transpeptidase (52). If net wall synthesis or wall turnover cannot explain these results then it is still possible that there is some rearrangement of wall structure that is necessary for sporulation septation or more probably its initiation. Alternatively, an effect of penicillin at the control level of septation has been postulated by Pitel and Gilvarg (67). It is interesting to note that spores, in contrast to vegetative cells, do not contain teichoic acids (9).

Understanding wall metabolism at stages I and II is important for understanding septation. One theory of septation is that wall synthesis stops and membrane

synthesis occurs resulting in "buckling" (67). That a rigid wall envelope is necessary is suggested by observations of sporangial protoplasts (24). A stage II protoplast consists of a large protoplast (future sporangium protoplast) linked with a tiny one (germ cell protoplast). Envelopment cannot occur under these conditions. Whether a stage I protoplast can undergo septation however is not clear. Division of vegetative cell protoplasts of B. megaterium has been reported (49) suggesting that a rigid wall may not be necessary for septation though the fact that such protoplasts still contained some wall components may imply that some cell wall is necessary for septation.

It is difficult to compare the mechanisms of cell division septation and sporulation septation with regard to cell wall growth. In rod-shaped cells wall extension and cross wall (septum) formation seem to be relatively independent processes (32). The concept of the unit cell developed by Donachie and Begg (18) is probably relevant to sporulation septation. With small cells of E. coli, at slow growth rates, elongation of the cell is in one direction only and is towards the youngest pole. In larger, faster growing cells, elongation occurs in two directions towards both poles. Presumably in sporulation, cell wall extension in at least one direction is repressed so that septation occurs without the normal preceding wall elongation. In this simplest model, septation (stage II)

occurs at what would have been the middle of the cell had elongation occurred. Freese (27) has considered 3 other models of asymmetric septation. Also cross wall formation in the septum is repressed though the preceding discussion does not rule out small but important rearrangements of the wall necessary for septation. In a sense, a sporulating rod represents a reversion of a dividing rod to the more primitive state of the dividing coccus (32) but without cross wall formation in the septum. As a result, the germ cell of the spore resembles a coccus.

An unanswered question with respect to stage I and II sporulation mutants is how they can exist without affecting normal cell division if they are indeed equivalent to modified stages of cell division. One answer is that such mutations are in a sense, conditional being expressed only under the metabolic conditions prevailing during the stationary phase. Such conditional sporulation mutants might be expected to be more frequently isolated than non-conditional mutants according to the hypothesis, for routine methods for selecting sporulation mutants depend on the mutant being able to form a population (84) by normal cell division. Thus the same genotype will be expressed as one of two different phenotypes according to the environmental conditions. For example, since the occurrence of the sporulation division process seems to depend on a shift-down in growth conditions, impairment

of the TCA cycle will affect sporulation division without necessarily affecting normal division. Hopefully such reasoning as this will help to explain how a mutant of B. subtilis isolated by Ito and Spizizen (40) and blocked early in sporulation (no extracellular proteases or antibiotic) lacked one membrane protein, had one modified membrane protein and yet could divide normally. They did not present these data as evidence against the cell division hypothesis which they seem to accept since they refer to "symmetric cell division" and "asymmetric cell division."

Phenethyl alcohol (PEA) is believed to affect the site(s) controlling DNA replication and cell division (117) though PEA also affects permeability and so may also affect DNA synthesis this way. Cell division septation and sporulation septation are both inhibited by PEA (70, 90). Sporulation septation is, however, more sensitive than cell division septation. Interestingly, Kretschmer (47) who confirmed this observation also found that stabilization of potential transformants of the preseptation stage in sporulation in B. subtilis is affected by the same concentration of PEA (0.3%). She suggests that PEA may be stabilizing some stage common to the two processes. The differential effect of PEA on division and sporulation is not unusual since sporulation is generally more sensitive to environmental influences than division (63).

Relevance of Division Modifications for Stages III-VII

Events subsequent to septation can be regarded as being facilitated by the modifications of the last cell division postulated to occur during stage I and II. Envelopment (stage III) of the small germ cell protoplast by the larger future sporangial protoplast can be regarded as the consequence of membrane synthesis being out of step with net cell wall synthesis which is delayed until stage IV (cortex formation). Since there is no wall elongation envelopment seems to be inevitable. The sporangium cell wall is necessary for envelopment (24). Envelopment is helped by the size differences between the two protoplasts and by the lack of a rigid cross wall in the sporulation septum.

Cortex formation (stage IV) involves peptidoglycan formation and it has been suggested that this is equivalent to cell wall formation by the larger protoplast (23). The cortical membrane is also peptidoglycan and can be regarded as germ cell primordial wall formation. In B. subtilis this primordial wall is formed before the cortex (75). The spore coat (stage V) is believed to be synthesized in the sporangium protoplast (64, 84, 92) since it is located there.

The end result of the modified cell division process is a dormant resistant germ cell encysted in products of

the sporangium, i.e. the coat and cortex. Most of the sporangium is destroyed by lysis (stage VII) thus freeing the encysted germ cell. Thus the imbalance and asymmetry that first becomes apparent during stage I and II is progressively amplified and in the end a major part of one of the cells resulting from the division is lyzed. The morphological changes that occur during sporulation seem to be principally the result of changes in the relative timing and quantity of synthesis of membrane and cell wall materials rather than changes in quality of the structural materials. This and other aspects of the modified cell division are reviewed in the Appendix.

The modified cell division hypothesis of sporulation provides a convenient framework on which to arrange consistently our knowledge of sporulation. Such an inductive approach, as the previous literature survey shows, cannot as yet explain every observation about sporulation. Many of the apparent contradictions will probably be settled as our understanding of this complex process increases.

Rod to Filament Morphopoiesis

As mentioned in the General Introduction, the availability of a thermosensitive mutant of B. megaterium which forms filaments at restrictive temperatures due to a defect in cell division septation made it possible to test a corollary of the hypothesis. The corollary is that mutants defective in cell division septation should be

asporogenic due to the defect also impairing the ability to make sporulation septa. In fact, there was already a suggestive precedent supporting this idea. Shaforostova (86) observed a dissociation of B. megaterium in continuous culture into normal rods and filaments with no cross walls visible by light microscopy. The dissociation occurred at intermediate mass doubling rates. The filamentous dissociant was stable and asporogenous. However, the stage at which sporulation was blocked and the possible presence of crosswall-less septa in the filaments were not checked by electron microscopy. In this thesis, these points have been checked with a mutant that has the added advantage of being temperature conditional. The results that were obtained are consistent with the postulated relationship between the two kinds of septation.

Bacterial filaments (synonyms "snakes" or "long forms") are elongated rod-shaped cells that contain no cross walls or septa. This definition of filaments excludes chains of cells but such a distinction is not always made in the filament literature. Filament formation can occur in Gram-positive and -negative rod-shaped cells. Rod-shaped cells are endowed with a cell lengthening mechanism, and filamentation is an exaggeration of this function due to inability to control it by means of septation. Spheroidal shaped cells or cocci, which do not have a well developed extension mechanisms, do not form filaments as easily.

Although long forms of cocci have been reported (32) they never reach the exaggerated lengths of filaments derived from rods. When rods lose their elongation capacity, as in certain mutants, coccoidal forms result (72).

Filaments sometimes occur naturally in growing cultures but with a low frequency. High-frequency filamentation can be induced by a variety of environmental and genetic effects (37). The filamentation effect of the inducing factor is sometimes cured by high sodium chloride concentrations, by pantoyl lactone or by photoreactivation. Diverse effects such as the presence of deleterious chemicals at low concentrations or nutritional deprivation cause filamentation. This suggests that division septa are readily dispensable cell structures and/or that their formation is the most sensitive physiological process in the cell.

Antibacterial agents that induce filamentation include dyes (37), antibiotics (29, 37), metabolite analogues and inhibitors (38, 83, 97), and heavy metals (37) including platinum compounds (74). Nutritional deficiences causing filament formation involve biotin (89, 94), iron (65), and magnesium and potassium ions (87, 110). On the other hand, filamentous forms are greatly reduced in slowly growing chemostat cultures (48) and filamentation of E. coli is induced in rich medium plus L-lysine (112). Physical effects such as

high (100) and low (88) temperature, radiation (42) or high pressure (118) can cause filamentation. Sometimes the cause of filamentation is not easy to define, e.g. spontaneous filamentation of Proteus at the edges of colonies (41).

Filamentation due to unconditional expression of genetic mutants is rarely studied (86). The most studied filamentation mutants are conditional mutants. The conditioning agents may be chemical (62) or physical agents such as ultra-violet irradiation (108, 113) and temperature (36, 103).

In Escherichia coli temperature sensitive (ts) filamentous mutants have been classified into two phenotypes but each phenotypic class contains several genotypes (36). One phenotype forms filaments with continued DNA synthesis and another forms filaments without prolonged DNA synthesis at the restrictive temperature.

The variety of factors inducing filamentation may reflect the apparent complexity of septation. This idea of complexity is supported by the mapping of filamentation mutants of B. subtilis (102) and E. coli (36, 99) at several widely separated loci.

One cause of filamentation appears to act on septation indirectly at the control level. Thus inhibition of DNA synthesis by a variety of treatments (ultra-violet irradiation, mitomycin C, thymine starvation, nalidixic

acid or certain conditional mutations) leads to filamentation (5). This is because septation cannot occur until a round of DNA replication is completed in E. coli. Replication completion signals the start of septation. The E. coli ts mutant of Reeve, Groves and Clark appears to affect this signal directly (68). However, some ts-DNA mutants continue to divide at restrictive temperatures thus yielding anucleate cells of uniform size (35) or of variable size (39). Since this division is inefficient, long filaments are eventually produced. Some of these continued division dna mutants may carry a second mutation responsible for this behavior (36).

A second cause of filamentation acts directly at the level of septation or at least cross wall formation. Thus low levels of penicillin can induce filamentation in E. coli, though sometimes the filaments are unstable unless high concentrations of salt are present (93). Penicillin induced filamentation appears to be related to the fact that the stage of the cell cycle most sensitive to penicillin is septation (59). Hartman, Höltje and Schwarz suggest this is because a critical enzyme (endopeptidase) probably concerned with cross-wall formation is more sensitive to penicillin than an equivalent enzyme (glycosidase) probably concerned with elongation (31). Decreased peptidoglycan content has been noted

as the result of nutritionally induced filamentations in E. coli (111, 112).

A third cause of filamentation appears to act directly at the membrane level. Ts-filament formation in E. coli due to a mutation at the azi locus is associated with increased azide and PEA resistance. PEA is believed to affect DNA membrane attachment sites (117).

Finally the possibility exists that some mutations may cause filamentation by affecting translation. A relationship between aminoacyl-tRNA synthetase activity and cell division has been reported (95, 96) in a ts-filamentation mutant of B. subtilis (56).

The ubiquity of agents inducing filamentation and the several known sites of action suggest that the process of septation is very complex. Thus, while filament forming mutants are tempting to study with respect to sporulation and cell division septations, the actual nature of the lesion causing aseptation is difficult to determine. Indeed, the mode of action of a filamentation mutation has not yet been worked out. Nevertheless, whether the mutation is intimately related to septation or its control or whether it is more distantly related perhaps via intermediary metabolism may be immaterial from the point of view of comparing cell division septation and sporulation septation. Thus although the relationship of the mutation and septation may be indirect it could still be important.

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ARTICLE 1

CELL DIVISION SEPTATION AND SPORULATION SEPTATION IN BACILLUS MEGATERIUM

By

A. D. Hitchins and H. L. Sadoff

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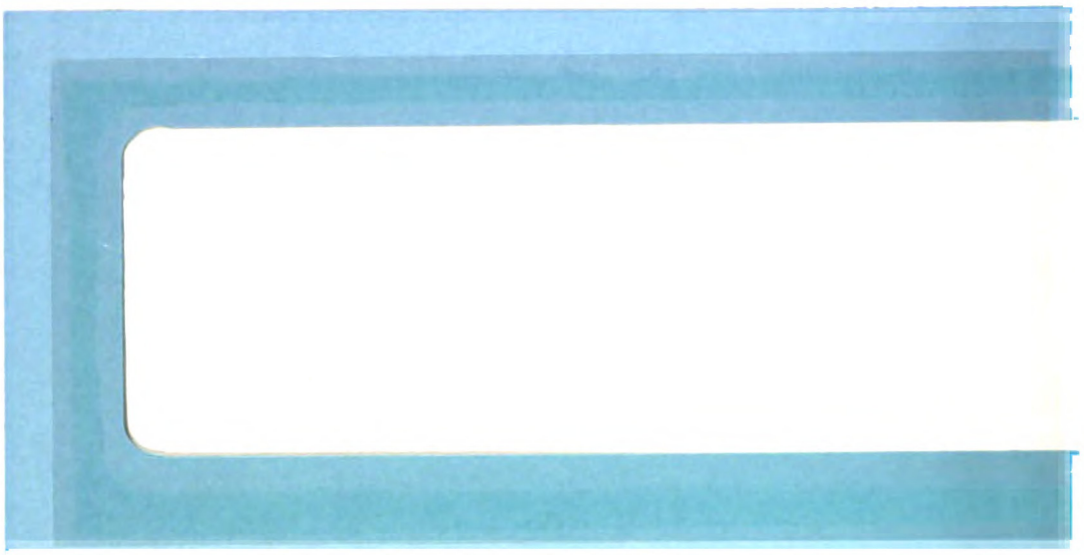
SPORES V

Cell Division Septation and Sporulation Septation in *Bacillus megaterium*[1]

ANTHONY D. HITCHINS AND HAROLD L. SADOFF

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823



SPORES V

Cell Division Septation and Sporulation Septation in *Bacillus megaterium*[1]

ANTHONY D. HITCHINS AND HAROLD L. SADOFF

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823

A temperature-sensitive mutant (TH 14) of *Bacillus megaterium* ATCC 19213 was isolated by replica plating colonies formed by survivors of nitrosoguanidine mutagenesis and a subsequent D-cycloserine selection step. The mutant and its parent grew at similar rates [mean generation time (MGT) about 2 hr] at 26 C, the permissive temperature. At 39 C, the restrictive temperature, the parent grew with an MGT of about 1 hr, but the mutant, after a short period of rapid growth, formed filaments with an MGT of 2 hr. The filaments were aseptate and, since deoxyribonucleic acid synthesis continued, were multinucleate. Filamentation was independent of the carbon source supplied for growth. The frequencies of sporulation of the mutant at 30 and 39 C were 0.6 and 10^{-5}, respectively, whereas the corresponding values for the parent were 0.8 and 0.1. No sporulation septa or later stages of sporulation were detectable in stationary-phase filaments. These and other results suggest that (i) the filamentation and asporogenic characteristics are due to the same mutant gene and (ii) the early stages of sporulation represent a modified cell division.

We are interested in comparing and contrasting the processes of cell division septation and sporulation septation and their control. The two processes have much in common, suggesting that the early stages of sporulation may represent a modified cell division. This idea was proposed by Robinow (8) in 1960 and has been reviewed (3, 4, 10). However, Kretschmer (6) has suggested that sporulation septation is due to the reinitiation of membrane synthesis at a polar zone that is normally responsible for cell elongation.

Sporulation septa differ from cell division septa in being asymmetrically located in the cell and in their lack of peptidoglycan. These two differences are important for the subsequent stages in development of a spore.

If the early stages of sporulation are analogous to cell division, then mutants defective in some phase of cell division [deoxyribonucleic acid (DNA) replication or its initiation, nuclear separation, septation] might be asporogenic due to blocks occurring before stage II of sporulation. We describe here the isolation and properties of a conditional temperature-sensitive (ts) mutant of *Bacillus megaterium* that is defective in septation (A. D. Hitchins and H. L. Sadoff. Bacteriol. Proc., p. 24, 1971). A similar ts filamentation mutant of *B. subtilis* has been reported (7), but it has not been used to compare cell division septation and sporulation septation.

[1] Publication number 5711 of the Agricultural Experimental Station, Michigan State University.

MATERIALS AND METHODS

Strains. *B. megaterium* ATCC 19213 was the parent strain used in this study. A temperature-sensitive mutant, TH 14, was derived from the parent strain, and a streptomycin-resistant TH 14 strain was also obtained.

Media. The defined sucrose-salts (SS) medium of Slepecky and Foster (11) was used, but in some experiments sucrose was replaced by other carbon sources. The concentrations of the carbon sources were 0.3% (w/v), and these were either autoclaved or filter-sterilized as appropriate. Nutrient broth and nutrient agar were used with or without a supplement of the trace metal salts of SS medium.

Cultures. Supplemented (metal salts) nutrient agar or SS medium agar slants were used for maintenance of all strains. Stock spore suspensions were prepared on SS agar or in SS liquid medium. Cultures were grown in 100-ml volumes of SS medium at 30 C from heated (70 C, 30 min) spore inocula. Inosine and alanine (25 μg/ml) were included to initiate germination, and growth was monitored by turbidity measurements at 600 nm in either a Beckman DU spectrophotometer (1-cm cuvettes) or a Beckman Spectronic-20 colorimeter (11-mm tubes).

Isolation of mutants. The following procedures were utilized to isolate the *B. megaterium* mutant TH 14. Cells were grown to mid-exponential phase in glycerol-salts medium at 30 C and then treated with 75 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per

ml (NTG; Aldrich Chemical Co.) for 30 min at room temperature. Approximately 5% of the cells survived this mutagenesis when counts were made on nutrient agar. The cells were washed twice in glycerol-salts medium, inoculated into fresh medium, and incubated at 30 C for 16 hr. This permitted the expression and multiplication of any conditional division mutants surviving the NTG treatment. A 10-ml sample of the culture was added to 50 ml of glycerol-salts medium and shaken for 2 hr at 37 C. After 2 hr of growth, D-cycloserine (100 μg/ml) was added to the culture to kill the dividing, nonmutant cells. This selection process was carried out over 4 hr after which a 5-ml sample of the culture was washed twice and inoculated into SS medium at 30 C to obtain spores. These were subsequently washed and stored as an aqueous suspension at 4 C.

Appropriate dilutions of the spore suspension were spread on glycerol-salts agar (L-alanine and inosine, 100 μg/ml each) to yield 100 colonies per plate at the permissive temperature. Two replicas of each plate were made on glycerol-salts agar without germinants. One plate of each pair was incubated at the permissive temperature, and the other was incubated at the restrictive temperature. The comparison of each pair of plates led to the isolation of six tentatively ts mutants out of about 1,500 colonies. Subsequent testing showed that five isolates, including TH 14, were ts on nutrient agar and one was ts only with glycerol as carbon source.

Light microscopy. Cells were examined and photomicrographs were taken with a phase-contrast microscope. The lengths of 25 or 50 cells per sample were measured with an ocular micrometer; one length unit is equivalent to 1,250 nm. Duplicate determinations of cell numbers were made with a Petroff-Hauser counting chamber. To 1 ml of cell suspension were added 0.2 ml of 10% formaldehyde and 0.8 ml of glycerol (50%, v/v). Samples were then diluted in glycerol (20%, v/v). Nuclei were stained by the Giemsa technique.

Electron microscopy. Culture samples of the mutant were collected on prewetted membrane filters (Millipore Corp.) and washed with 10 ml of glutaraldehyde (3%) in 0.1 M sodium cacodylate (Sigma)-HCl buffer, *p*H 7.2. A 1-mm layer of warm Noble agar (1%) was poured over the filter. The filter with agar was kept in cold glutaraldehyde (3%) and buffer overnight. After being washed three times with buffer over a period of several hours, the filter with agar was cut into small squares. Specimens were postfixed in the osmium tetroxide fixative of Kellenberger, Ryter, and Séchaud (5) for 19 hr at room temperature. The specimens were posttreated for 2 hr with 0.5% uranyl acetate in Kellenberger's buffer and then dehydrated as follows: 50, 70, and three changes of 100% ethanol followed by three changes of propylene oxide. Dehydrated specimens were taken through a graded series of propylene oxide-Epon mixtures into pure Epon. After polymerization, the samples were sectioned with a diamond knife on a Porter-Blum MT-2 (Sorvall Inc., Norwalk, Conn.) ultramicrotome. Sections were mounted on 300-mesh copper grids and viewed with a Philips 300 electron microscope at an accelerating voltage of 60 kv. The fine structure of the parent strain cells (grown at 30 C in SS medium) has been reported (2).

DNA. DNA was determined by both the methods of Ceriotti (1) and of Roodyn and Mandel (9). For the latter determination, the procedure of Yang and Brubaker (12) was followed except that 1.79 mM uracil-2-^{14}C (Calbiochem; 0.056 μCi/μmole) was used. Sample sizes were reduced (1.0 to 0.5 to 0.2 ml) with increasing cell densities to facilitate filtration.

Viable counts. These were determined by spreading 0.1-ml samples of dilutions on nutrient agar. Heat-resistant colony-forming units were determined by heating samples (70 C, 30 min) in totally submerged sealed glass ampoules.

RESULTS

Filamentation. The temperature-sensitive mutant of *B. megaterium* designated TH 14 formed filaments at 37, 39, and 41 C, whereas the parent strain grew normally producing rod-shaped cells at these temperatures. At 26 or 30 C, the mutant cells were normal in appearance. Occasionally filaments occurred at 30 C but only at a low frequency (less than 10^{-2}).

Substrates. At restrictive temperatures, filamentation occurred in nutrient broth or in defined medium containing a single carbon source. The substrates tested were: sucrose, sodium citrate, ribose, L-glutamate, glycerol, sodium succinate, L-alanine, sodium pyruvate, and sodium acetate.

Growth. The mutant and parent strains had mean generation times (MGT) of 2 hr at 26 C and 1.5 hr at 30 C in SS medium. At 39 C, the parent strain had an MGT of 1 hr in the same medium. When the mutant strain was grown at 26 C and then was shifted to 39 C, its growth rate (measured by turbidity change) increased for about the first hour but then gradually returned to the rate characteristic of 26 C (Fig. 1). Short filaments were detectable by the third hour after shift-up, and these increased in length with continued incubation (Fig. 2).

Cell size and number. After a temperature shift-up, the average cell length of mutant TH 14 began to increase at 2 hr, but the growth response appeared to be biphasic (Fig. 3). There was a rapid initial increase which lasted for 2 to 3 hr, but thereafter the total count increased more slowly. The frequency distribution of cell sizes showed that the filament population is very heterogeneous with regard to size and that 1 to 10% normal size cells are always present (Table 1). The frequency and total count data showed that some cell division of mutant TH 14 may be possible at the restrictive temperature.

DNA synthesis and septation. DNA synthesis occurred throughout filamentous growth (Fig. 1).

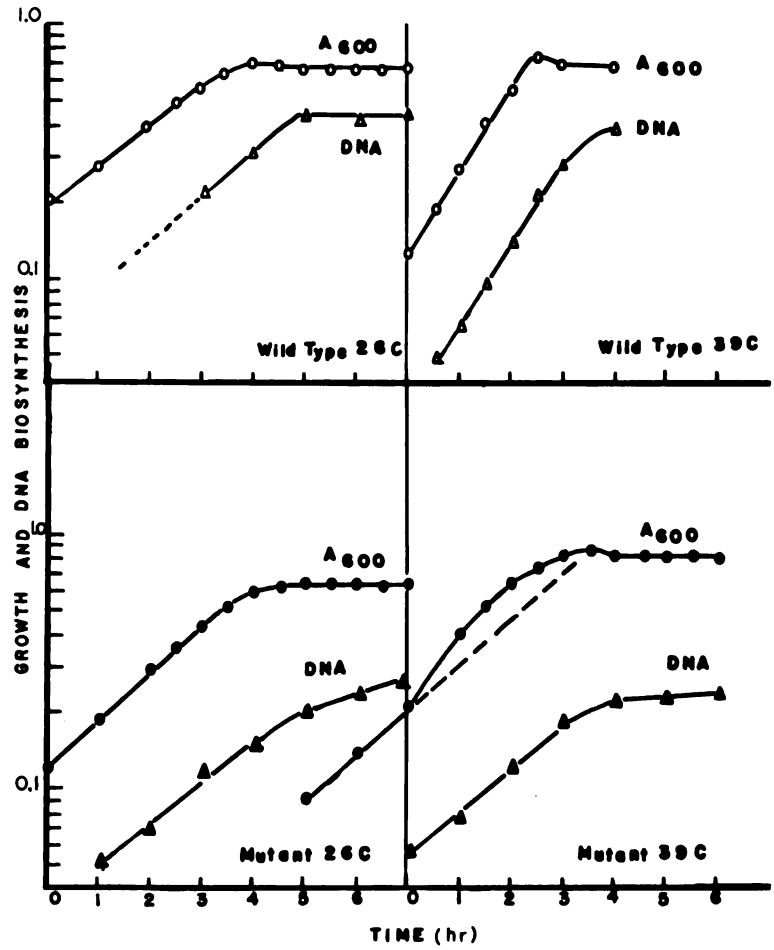


FIG. 1. *Growth and DNA biosynthesis by wild type and ts mutant TH 14 at 26 and 39 C. The SS medium (200 ml) contained 0.1% (w/v) sucrose. Culture turbidity was measured at 600 nm. DNA was measured by the indole method (1) and plotted as absorbancy (A) units at 490 nm. One A_{490} unit is equivalent to 5 μg of DNA per ml of culture. Culture samples (20 ml) were added to 0.8 ml of 12.5 N perchloric acid at 4 C to stop growth. Symbols:* ○, *growth of wild type;* ●, *growth of mutant;* △, *wild-type DNA;* ▲, *mutant DNA.*

Multiple nuclei could be seen in filaments stained by the Giemsa method. Electron microscopy of ultrathin sections of filaments at various stages of development confirmed that filaments are multinucleate and aseptate (Fig. 4) and that no abnormal forms of septation occurred.

Temperature shift-down and subculture. When filamentous cells at the restrictive temperature were shifted-down to the permissive temperature, division did not begin until an hour after shift-down (Fig. 5). The overall effect of subculture of filamentous cells by dilution into prewarmed fresh medium is that filaments increased in length. However, first there was a lag in the average length increase followed by a decrease.

Viability and cross feeding. Table 2 shows that the colony-forming ability of spores of the mutant TH 14 decreased markedly at temperatures above 30 C, whereas that of wild-type spores was little affected. The cell viability of mutant TH 14 had a temperature response similar to that of its spores. Colonies formed by the mutant at the restrictive temperature grew slowly and remained small compared to wild-type colonies. No cross-feeding effect was observed at 37 or 41 C between mutant and wild-type strains.

Sporulation. The frequency of sporulation of wild-type and mutant strains is shown in Table 3. The mutant was asporogenous at restrictive temperatures even when allowance was made for the decreased sporulation of the wild-type strain at such temperatures. The heat-resistant colony-

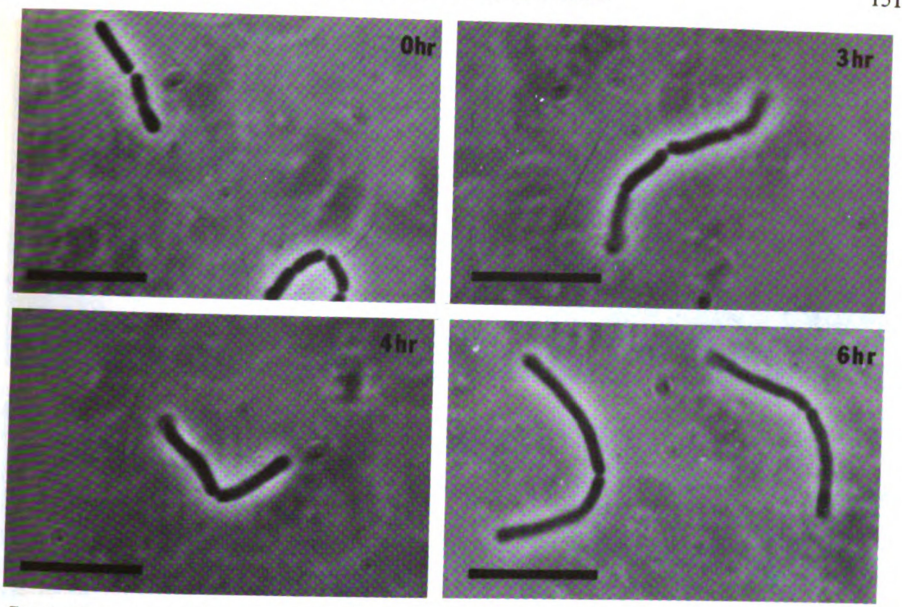


FIG. 2. *Phase-contrast photomicrographs of filament formation by ts mutant TH 14 at 39 C in SS medium. Times indicate hours after shift-up to 39 C. The scale bars represent 15,000 nm.*

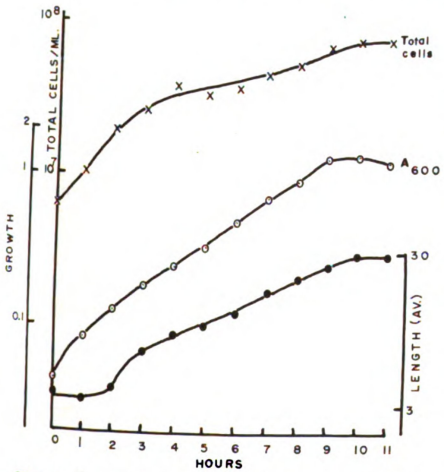


FIG. 3. *Formation of filaments by ts mutant TH 14 in SS medium. The culture was shifted from 30 to 39 C at time zero. Symbols: ○, culture optical density at 600 nm; ×, total cell count per ml; ●, average cell length (1 unit equals 1,250 nm).*

forming unit count suggests that the mutant is not totally asporogenic sensu strictu but is weakly oligosporogenic.

Electron microscopy of ultrathin sections of stationary-phase filaments showed that they lack sporulation septa or any other later stages of sporulation. A comparison of the DNA yields of permissive (sporulating) and restrictive (asporogenic) cultures of the mutant suggests that the asporogeny is not due to an inability to complete the last round of DNA replication which forms the mother cell and germ cell genomes.

TABLE 1. *Change of frequency[a] distribution of cell lengths with incubation time at 39 C*

Time (hr)	Cell length category[b]							
	1–5[c]	6–10	11–15	16–20	21–25	26–30	31–35	36–51
0.0	100	0	0	0	0	0	0	0
1.3	98	2	0	0	0	0	0	0
3.0	58	42	0	0	0	0	0	0
5.0	12	60	26	2	0	0	0	0
7.0	4	20	34	26	12	4	0	0
9.5	8	8	18	20	10	16	10	10

[a] Frequency distribution is expressed in per cent; 50 cells equal 100%.

[b] Cell length is measured in arbitrary units (1 unit equals 1,250 nm).

[c] Parent cells and permissively grown cells of TH 14 fit into the 1–5 category.

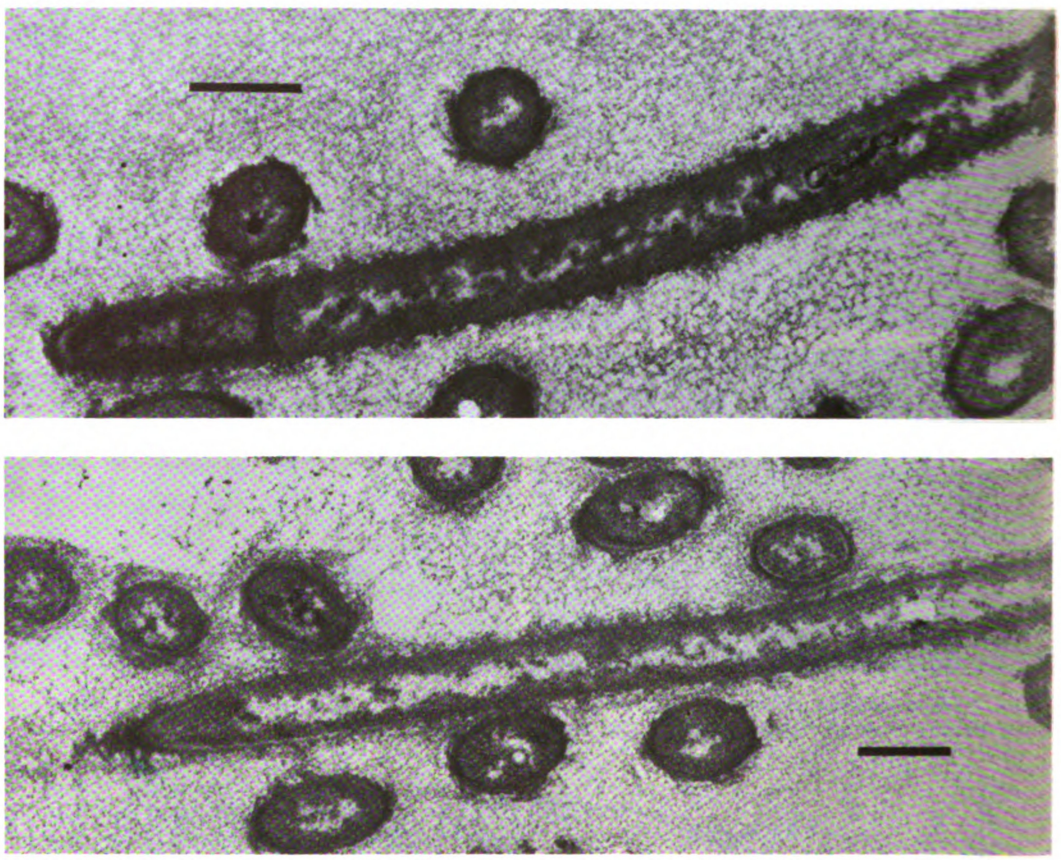


FIG. 4. *Electron micrographs of ultrathin sections of stationary-phase filaments of mutant TH 14. The scale bar represents 1,000 nm. The micrographs show that the filaments are aseptate and multinucleate. One of the filaments is attached to a cell.*

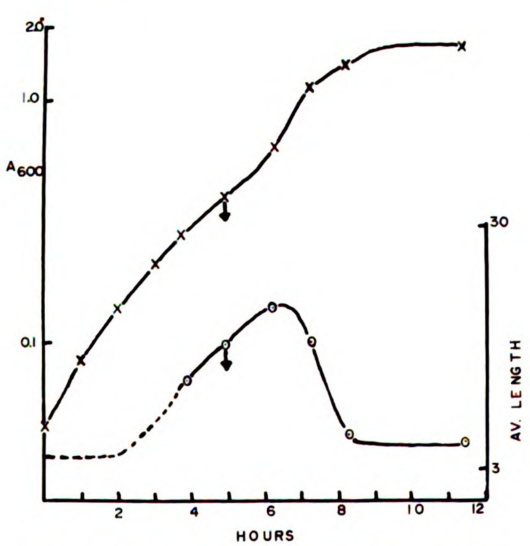


FIG. 5. *Effect of shift-down of the growth temperature from 39 to 30 C on filaments. The arrows indicate time of shift down. Symbols: ×, growth; ○, average cell length. One length unit equals 1,250 nm.*

The shift-down of stationary-phase filaments to permissive temperatures results in the formation of a low percentage of sporangia. Only the shorter filaments form such sporangia and in this event the spores are terminally located. Preliminary work with six revertant isolates, appropriately

TABLE 2. *Effect of growth temperature on the viability of spores and cells of the parent and mutant strains*

Determination	30 C	37 C	39 C	41 C
Spare viability[a]				
Parent	100	96.0000		87.00000
Mutant TH 14	100	1.0000		0.00003
Cell viability				
Mutant TH 14	100	0.2800	0.0080	
Mutant TH 14 *str*^{r}	100	0.0008	<0.0001	

[a] Viability is expressed as per cent survivors, the value at 30 C being taken as 100.

TABLE 3. *Sporangia frequency*[a] *of parent and mutant strains at various growth temperatures*

Determination	30 C	38 C	39 C	42 C
Parent.....	78 (9)[b]	47 (11)	7 (7)	0 (0)
Mutant TH 14.....	57 (14)	0 (0)	0 (0)[c]	0 (0)

[a] Sporangia frequency expressed as percentage of cells containing phase-bright spores.

[b] Values in parentheses are percentages of cells containing phase-dark or incompletely phase-bright spores.

[c] Heat-resistant colony-forming count equals 108 per ml of culture.

marked with streptomycin resistance, showed that reversion to temperature resistance is accompanied by reversion to sporogeny.

DISCUSSION

The *B. megaterium* TH 14 ts mutant forms multinucleate, aseptate filaments and has a reduced rate of growth at restrictive temperatures. Asporogeny is associated with this phenotype, and thus these two properties probably result from the same mutation. The asporogenic property was not utilized for selection and isolation of the mutant. The asporogenic phenotype is ts like the filamentous phenotype, and the loss of the ts filamentation property, by spontaneous reversion, results in the simultaneous reappearance of the sporogenic character. The slight "leakiness" of both the filamentation and asporogenic properties is consistent with this genetic relationship.

The ts mutation may be in a gene that functions in both cell division septation and sporulation septation, but the role of the gene product is unknown. It could act at either the control or structural level. The results which are presented support the idea that early stages of sporulation are equivalent to a modified cell division.

ACKNOWLEDGMENTS

We thank Prudence J. Hall and H. Stuart Pankratz for help with the electron microscopy.

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ARTICLE 2

FURTHER PROPERTIES OF A THERMOSENSITIVE ASPOROGENOUS FILAMENTATION MUTANT OF BACILLUS MEGATERIUM

By

A. D. Hitchins and H. L. Sadoff

(Manuscript in preparation)

Further Properties of a Thermosensitive Asporogenous Filamentation Mutant of *Bacillus megaterium*[1]

ANTHONY D. HITCHINS AND HAROLD L. SADOFF

Department of Microbiology and Public Health,
Michigan State University,
East Lansing, Michigan, 48823

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(ABSTRACT)

Thermosensitive mutant, TH14, of Bacillus megaterium ATCC19213 is defective in cell division septation and spore formation at the restrictive temperature (39 C). As a consequence, the mutant forms multinucleate aseptate filaments and is asporogenic. The mutation does not result in any qualitative compositional changes in membrane proteins which are extractable in hot sodium dodecylsulfate plus 2-mercaptoethanol. At the restrictive temperature, the mutant has a reduced content of a small molecular weight protein(s) equivalent in size to cytochrome c. A membrane protein(s) with a molecular weight of nearly 80,000 appears to be partially derepressed in the mutant grown at restrictive temperature. In addition, numerous unidentified spherical inclusions of fairly uniform size (diameter circa 100 nm) are present in the cytoplasm at the restrictive temperature. Filamentous growth of the mutant is less sensitive to penicillin than growth in the rod form. Growth in either form is equally sensitive to D-cycloserine at the concentrations used for selection of the mutant. Temperature shift-up experiments suggest that 1 to 2 rounds of deoxyribonucleic acid (DNA) replication occur before the phenotypic expression of the mutation

occurs. The septations corresponding to the DNA replications can be either 2 division septations or 1 division septation plus a subsequent sporulation septation. This conclusion coupled with previously reported work supports the hypothesis that the early stages of sporulation represent a modified cell division.

Bacterial cell division mutants whose expression is thermosensitive (ts) have proved to be useful for understanding division (1,14,15,23,26,28). In contrast, although it is about 12 years since ts bacterial sporulation mutants (Spo ts) were first isolated by Lundgren and Beskid (18), use of ts mutants in studying sporulation has been rare (30). Nevertheless, the ability to control the phenotypic expression of a mutant gene by temperature shifts is particularly useful in studying the role of that gene in processes consisting of sequential events such as sporulation (11). This is especially true when the gene's function is known.

Recently, two ts cell division mutants have been used to study sporulation. One of these is a mutant with ts initiation of deoxyribonucleic acid (DNA) synthesis (20). The other, which is the subject of this report, is a ts mutant (TH 14) of Bacillus megaterium which grows normally at 30 C but forms aseptate filaments at 39 C, the restrictive temperature. This mutant is also ts asporogenous being blocked at sporulation Stage I (DNA

in axial filament configuration) since no sporulation septa (Stage II) are detectable at restrictive temperature (10). Apparent lack of recognizable Stage I forms in the mutant at the restrictive temperature can be attributed to the difficulty of defining the stage in the parent strain (6). According to the recently recommended terminology of Young and Wilson (34) ts mutant TH14 can be designated Spo ts I with respect to its ts asporogenic property.

Thermosensitive blocking of cell division septation and sporulation septation in the same mutant suggested that the two processes are closely related (10). Such a relationship supports the hypothesis that the early stages of sporulation (Stages I and II) represent a modified cell division (11). In this paper we compare the membrane proteins of the parent strain and the mutant at permissive and restrictive temperatures (A. D. Hitchins and H. L. Sadoff. Bacteriol. Proc., p. 37, 1972) and report some other properties of the mutant.

MATERIALS AND METHODS

Bacterial strains. The parent strain was Bacillus megaterium ATCC19213. The mutant strain (TH14) was derived from the parent strain by chemical mutagenesis (10).

Previously described techniques. Determinations of heat resistant colony forming units, percentage of sporulation and deoxyribonucleic acid (DNA) biosynthesis and the preparation of ultrathin sections for electron microscopy are described in the preceding communication (10).

Culture conditions. Cell and filament-form cultures for isolation of membrane proteins were prepared by allowing preheated spores (10^8/ml; 70 C for 30 min) to germinate and grow out at 30 C and 39 C respectively. Spores were produced on solid medium as described previously (10). The medium used was the SS-medium of Slepecky and Foster (29) containing sucrose (0.3% w/v) and supplemented with germinants (L-alanine and inosine, 100 µg/ml each) and L-leucine (50-300 µg/ml). Cells and filaments were harvested by centrifugation (10,000 x g for 30 min) when the culture turbidity (corresponding to an absorbancy reading at 600 nm) was 0.7 to 1.0 (equivalent to about 3×10^8 to 4×10^8 cells/ml). Drained cell and filament pellets from 100-ml cultures were stored at -10 C. When

labeled membrane proteins were required, 100-ml amounts of the above medium were further supplemented with either L-leucine-4, 5 ^{3}H (1 mCi; 30-50 Ci per mmole) or L-leucine-U-^{14}C (50 µCi; 222 mCi/mmole). Cultures for other experiments were prepared using vegetative inocula grown as described previously (10).

Membrane preparation. Protoplast ghosts were prepared by lysozyme lysis of rods or filaments with lysozyme under hypotonic conditions. The method was based on that of Salton and Freer (25). Frozen pellets of rods or filaments (equivalent to 3 to 4 x 10^{10} cells) were thawed and resuspended in 10 ml Tris-HCl buffer (trishydroxymethylaminomethane, 0.05 M and pH 7.5 at 30 C) containing protease inhibitors (ethylenediaminetetraacetic acid and phenylmethylsulfonyl chloride both at a concentration of 10^{-3} M) and eggwhite lysozyme (E.C. 3.2.1.17; 250 µg/ml). Viscosity increased due to release of DNA from the lysing cells. Viscosity was decreased by addition of beef pancreas deoxyribonuclease (E.C. 3.1.4.5; 200 µg/ml). After 1 to 1.5 hr protoplast ghosts were sedimented by centrifugation at 10,000 x g for 30 min. The supernatant containing the cell cytoplasmic fraction was stored at -10 C. Protoplast ghosts were resuspended in 5 ml Tris-HCl buffer (0.05 M and pH 7.5 at 5 C) and separated from unlysed cells and particulate debris by differential centrifugation in 12-ml tubes in a HB4 swing-out head of a Sorvall centrifuge at

5,000 x g for 1 min. The resulting pellet from each differential sedimentation was resuspended in 5 ml buffer and recentrifuged. The supernatant solutions from the initial centrifugation and three subsequent runs were pooled. The final pellet which was mainly whole cells and debris was discarded. The pooled supernatant solutions were centrifuged at 40,000 x g for 30 min. The protoplast ghost pellet was resuspended and washed twice by centrifugation in fresh cold Tris-HCl buffer. The final protoplast ghost pellet was drained and stored overnight at -10 C or immediately treated with membrane dissolution mixture.

Membrane dissolution. Washed protoplast ghost pellets (membrane equivalent to that from about 4 x 10^{10} cells) were suspended in 0.3 ml of membrane dissolution mixture. This mixture contained sodium dodecylsulfate (SDS; 1% w/v) and 2-mercaptoethanol (2% v/v) in sodium phosphate buffer (0.01 M and pH 7.0). The ghosts were heated in this mixture at 95 C for 10 min. Insoluble material was removed by centrifugation at 10,000 x g for 20 min at room temperature. The supernatant containing extracted membrane proteins was stored at room temperature in airtight vessels.

SDS-polyacrylamide gel electrophoresis (PAGE). The SDS-PAGE method for analysis of membrane proteins was based on published methods (27,33). The gel polymerizing mixture, in glass tubes (100 x 6 mm internal diameter), contained

acrylamide (10% w/v), N,N'-methylenebisacrylamide (0.368% w/v), ammonium persulfate (0.07% w/v), sodium phosphate buffer (0.1 M and pH 7.0), SDS (0.1% w/v) and N,N,N',N'-tetramethylethylenediamine (0.03% v/v). The electrophoresis buffer consisted of sodium phosphate buffer (0.1 M and pH 7.0) containing SDS (0.1% w/v). Gels were prerun at 4 ma/gel tube for 1 hr to remove ammonium persulfate and other low molecular weight materials.

Samples applied to each gel contained 1 µl of tracking dye (Bromphenol blue; 0.1% w/v), 1 drop of glycerol and 5-15 µl of the membrane protein solution (protein content 8-16 mg/ml). Protein was determined by the method of Lowry et al. (17). Electrophoresis was run at 8 ma/gel until the tracking dye front reached the end of each tube. Gels were stained for protein with Coomassie brilliant blue by the method of Weber and Osborn (33) except that destaining was done by diffusion at 37 C into repeated changes of destaining fluid. Stained gels were scanned using a Gilford densitometer at a wavelength of 600 nm. Bovine serum albumin, fraction V (68,000 molecular weight), and horse heart cytochrome c (11,700 molecular weight) were used as size marker proteins (33).

Gels containing mixtures of membrane proteins labeled with L-leucine 4,5-^{3}H and L-leucine-U-^{14}C respectively were frozen at -10 C and sliced into 1-mm discs using a multiple razor blade gel slicer (Diversified

Scientific Instruments, San Leandro, Calif.). Slices of gel were digested with 0.2 ml hydrogen peroxide (30% by volume) at 70 C for 12 hr in glass scintillation vials. The dry residues were dissolved in 0.2 ml of deionized distilled water. Bray's (4) scintillation fluid (5 ml) was added and the mixture shaken well. Vials were counted for 10 min in a Packard Tri-Carb scintillation spectrometer (model 3320). Tritium was counted at a gain of 61.5% while ^{14}C was counted at a gain of 11%. The over-spill of the tritium count into the carbon channel was about 1% of the tritium channel count and that of ^{14}C into the tritium channel was about 3% of the carbon chennel count. In calculating $^{14}C/^{3}H$ ratios or *vice versa* the relatively insignificant over-spill was ignored.

Sources of chemicals. Antibiotics were obtained as follows: sodium penicillin G and D-cycloserine (Nutritional Biochemicals Corp., Cleveland, Ohio), rifampicin (Mann Research Labs., New York, N. Y.) and chloramphenicol (Parke Davis and Co., Detroit, Mich.). The sources of the proteins used were: lysozyme and beef pancreas deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo.), horse heart cytochrome *c* (General Biochemicals Inc., Chagrin Falls, Ohio) and bovine fraction V albumin (Nutritional Biochemicals Corp.).

L-alanine and inosine were obtained from CalBiochem, San Diego, Calif. and L-leucine from Nutritional Biochemicals Corp. L-leucine-4,5-^{3}H was from International Chemical and Nuclear Corp., Waltham, Mass. and L-leucine-U-^{14}C was from CalAtomic, Los Angeles, Calif. The following chemicals were bought from Eastman Organic Chemicals, Rochester, N. Y.: ethylenediaminetetraacetic acid (EDTA), acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine and 2-mercaptoethanol. Other chemicals were obtained as follows: phenylmethylsulfonyl chloride (CalBiochem), sodium dodecylsulfate (Sigma Chemical Corp.), trishydroxymethylaminomethane (Nutritional Biochemicals Corp.), ammonium persulfate (Fisher Scientific Co., Fair Lawn, N. J.), bromphenol blue sodium (Allied Chemical, New York, N. Y.) and Coomassie brilliant blue R-250 (Mann Research Labs.).

RESULTS

Spore germination and outgrowth. Fig. 1 shows the germination and outgrowth of mutant and parent spores at permissive (30 C) and restrictive (39 C) temperatures. Initiation of germination of the spore suspension, which occurs during the initial decline of turbidity, was slightly slower with the mutant at 39 C than in the parent at 39 C. The times for the turbidity values to decrease to 50% of the initial value were 10 and 5.5 min respectively. As expected from the results of growth experiments (10), mutant spore outgrowth and growth at the restrictive temperature were thermosensitive and resulted in filamentation. Also predictable from the results of previous experiments (10), the mutant grew slower at 39 C when forming filaments than at 30 C when growing in the rod form. Thus, the turbidometrically measured mean generation time (MGT) of the mutant was 1 hr at 30 C and 2 hr at 39 C. In contrast, the parent strain had a similar MGT (about 1 hr) at both temperatures. In rod-form cultures the second division was occurring by the time the turbidity had reached a value of 0.7.

Membrane proteins. Preliminary electrophoresis studies employing gels stained with Coomassie blue showed

that at least 25 protein fractions were contained in the solubilized membrane preparations. No qualitative differences between the protein fractions of parent and mutant membranes at permissive and restrictive temperatures (Fig. 2) could be detected by visual examination and spectrophotometric scanning of the stained gels. However, one protein fraction (A) with a molecular weight of about 80,000 was increased in amount in the mutant at the restrictive temperature relative to other proteins. Another fraction (Z) was decreased in amount in the mutant at the restrictive temperature.

The preliminary observations with stained gels were confirmed by SDS-PAGE fractionation of three appropriate combinations of differentially labeled (^{14}C and ^{3}H) membrane proteins prepared from parent and mutant cells grown at permissive and restrictive temperatures. The double labeling profiles obtained in four experiments are shown in Figs. 3, 4, 5 and 6. Table 1 summarizes the results from 5 profiles obtained using 3 different mixtures. The results are expressed as the average radioisotope ratios of all fractions and 3 subfractions (A, Z and all fractions except A and Z). Table 2 shows the radioisotope ratios of the A and Z fractions relative to all other fractions as calculated from the results in Table 1. Variation of the relative amounts of either the A or the Z fraction in the parent and the mutant strains

at permissive or restrictive growth conditions is shown in Table 3. The relative amounts of the A and the Z fractions were calculated from the data in Table 2. In Table 3, it can be seen that both the A and the Z fractions increased by about 10% in the parent stain when the temperature was changed from the permissive to the restrictive condition. In contrast, when the corresponding change was made with the mutant strain, the A fraction increased by about 50% while the Z fraction decreased by about 30%. Fraction A was about the same molecular weight as horse heart cytochrome c (11,700).

Inclusion bodies. Amorphous inclusion bodies were found in the filaments formed by the mutant at restrictive temperature. The bodies were circular in cross section with fairly uniform diameters of about 100 nm. This suggested they were spherically shaped. They are illustrated in Figs. 7 and 8. The bodies were relatively electron transparent suggesting that they did not contain material with uranyl ion binding sites. The bodies were detectable within 3 hr after shift-up of cells to the restrictive temperature but may be formed earlier than this. A notable feature of the inclusions was their relatively large concentration at one of the poles of the filaments.

Antibiotic sensitivity. Fig. 9 shows that filaments growing at 39 C were more resistant to penicillin than normal cells of the mutant growing at 26 C. No such

difference was detectable with D-cycloserine at concentrations of 25, 50 and 100 µg/ml which totally inhibited growth. Rifampicin (1, 10 and 50 µg/ml) inhibited growth of filaments and rods of the mutant totally. Preliminary results suggest that chloramphenicol (10 µg/ml) inhibited division of filaments after shift-down of growth temperature.

Temperature shift-up. Fig. 10 shows the effect of temperature shift-up on sporulation of samples taken at different times during the exponential and stationary phases of growth of a culture at permissive temperature. Temperature shift-up before 40% of total growth had occurred inhibited by 80% the formation of microscopically detectable spores. Shifts-up during the time when growth increased from 40 to 70% of the total growth allowed an increasing proportion of spores to be produced. During the mid-stationary phase of growth, temperature shifts (increases) again caused a decline in spore formation. When a temperature shift-up was carried out on cultures that had reached 40 to 100% of the total possible growth, the spores formed were less heat resistant than those produced after a shift-up late in the stationary phase. The heat-resistant colony-forming unit curve confirms the mid-stationary phase decline in postshift-up spore formation shown by the percent spores curve (Fig. 10). Maximal postshift-up sporulation was observed when the temperature change occurred in the late exponential, early stationary and late stationary growth phases.

DNA synthesis. DNA synthesis continued during filamentation at 39 C. Fig. 11 shows growth and DNA synthesis by the mutant strains at the permissive (26 C) and restrictive (39 C) temperatures. The extent of growth and the yield of DNA were the same whether conditions were restrictive or permissive. After temperature shift-up the rates of both growth and DNA biosynthesis were increased by about the first hour. Thereafter both the rates of growth and DNA synthesis decreased to the corresponding permissive rates.

DISCUSSION

Despite the general similarity between the membrane proteins of the parent and mutant strains, two well defined differences were noted. In comparing the proteins several assumptions were made. (1) It was assumed that the proteins were equally extractable from the membranes of either strain grown at permissive or restrictive conditions. The extraction procedure was rigorous and commonly used in these kinds of comparisons (7,14,26). (2) The complete disaggregation of protein subunits was assumed. The high extraction temperature used should have helped to ensure this but could have produced some artifacts due to the scission of covalent peptide bonds (26). (3) It was assumed that the extent of quenching of fluorescence was constant in all SDS-PAGE protein fractions (26). Variations in quenching would have had the greatest effect on the assay of tritium-labeled membrane proteins. The similarities between the distribution of radioactivity (both ^{3}H and ^{14}C) and the scans of the stained electropherograms suggest that quenching was constant in the various fractions. (4) It was assumed that no membrane proteins were lost during membrane isolation procedures. The method of Salton and Freer

(25) was selected because it minimized such losses. They found that protoplast ghosts of Gram-positive bacteria (including Bacillus sp.) prepared in Tris-HCl buffer retained their carotenoids and cytochromes much better than ghosts prepared in phosphate buffer.

As expected, electrophoresis resolved the soluble membrane preparation into a large number of fractions. An outstanding feature of the SDS-PAGE protein profiles was that one component (fraction Z) formed 50 to 60% of the total extractable membrane protein (Fig. 3, 4, 5 and 6). A major component of such proportions is not seen in most published membrane protein profiles (7,14,15,26,28). However, some of the profiles of Siccardi et al. (28) do resemble those reported here. Presumably, the protein profile would be dependent on the method of protoplast ghost membrane preparation and the method of Salton and Freer (25) was not used for the published profiles referred to above (7,14,15,26,28). It is unlikely that the fraction Z is due to heat induced rupture of peptide bonds during extraction since its ^{3}H and ^{14}C ratio differs from the average ratio. To achieve such a result, the rupture would therefore have had to be differential with respect to ^{3}H and ^{14}C labled proteins. Another possibility is that membrane proteins were lost during the protoplast ghost preparation due to the presence of EDTA (10^{-3} M) in the Tris-HCl buffer. Such treatment is known to osmotically unstabilize membranes by removing divalent cations such as magnesium (19). However, in the absence of evidence to the contrary, it is unlikely that EDTA at

10^{-3} M would cause massive and selective removal of proteins from membranes. Therefore, it must be assumed that the Z fraction represents a small protein or a protein subunit present in high concentration in the membrane of B. megaterium.

Fraction Z was relatively repressed in the mutant at the restrictive temperature (Table 2). The protein(s) in the fraction corresponds in size to cytochrome c but whether the fraction is homogeneous has not been determined. If it were cytochrome c, the interesting possibility would arise that its repression in the mutant at the restrictive temperature could be due to iron limitation, perhaps due to some defect in iron transport. Iron transport in the particular strain used in this study is known to be mediated by the siderochrome, schizokinen, a hydroxamate of citric acid (22). Iron limitation has been reported to cause filamentation (24) but these observations have been disputed (2). Cytochrome (a, b, c) repressed or deficient mutants of B. subtilis are asporogenic (31). However, such mutants are not filamentous. Sometimes the effect on the cytochromes in such mutants is a secondary result of blocks in the tricarboxylic acid cycle or of thiamine deficiency. Again, these latter mutants are not filimentous.

A reduced cytochrome c content in cells could conceivably cause energy limitation due to a decreased rate of oxidative phosphorylation. This could account for the reduced growth rate of the mutant at the restrictive

temperature. It seems improbable, however, that energy limitation could act selectively at the level of cell division septation though DNA synthesis in aerobically growing Escherichia coli is stimulated by uncouplers of oxidative phosphorylation (12).

The membranes were obtained from cells and filaments which had been harvested at a time when only 50 to 70% of the total possible growth had occurred. This variability in time of harvest could have affected the cytochrome content of the membrane, for it is known that the cytochrome (a, b, c) content of B. cereus cells increases markedly at the end of growth (16).

Fraction A was relatively derepressed in the mutant grown at the restrictive temperature. It is not known whether fraction A was homogenous and heterogeneity would partially mask more marked changes in a given protein's concentration.

Though changes in fractions A and Z were the most noticeable it is possible that changes in the levels of other membrane proteins occurred which were not easily resolvable from the background noise of such experiments. Changes in A and Z were readily detectable because of their relative concentration or because of their more obvious locations in the gels near the origin and tracker dye front respectively.

The differences in membrane composition between filaments and rods could be due to (a) the direct effect of the filamentation mutation on a control or structural gene, or (b) due to pleiotropic effects. One possible pleiotropic effect would result if there were differences in the protein composition of lateral membrane and septum membrane. Lack of septum membrane would result in absence of septum specific proteins. This possibility cannot apply to a fraction like Z which forms such a major part of the total membrane protein. Neither could it apply to protein fraction like A which increases at the restrictive temperature.

The spherical inclusion bodies were observed in mutant cells which had been grown at the restrictive temperature. They appear to be due to the filamentation mutation. Their chemical composition is unknown. They could be poly-beta-hydroxybutyrate (PHB) granules but if so, do not resemble normal PHB granules. PHB is the reserve material of this organism and PHB granules were visible in the filaments by phase-contrast microscopy but they were not concentrated at the polar regions of filaments like the granules.

The granules do not resemble DNA. They probably do not contain ribonucleic acid (RNA) like the aggregates occurring in platinum-compound induced filaments of E. coli (13) since they are relatively electron transparent.

Electron microscopically, they do not resemble the protein-containing bodies described in a ts mutant and a normal strain of two Bacillus species (3,5). The greatest concentration of inclusions occurred at the polar regions of the filaments. There was some indication that they may be localized only at one pole. However, this has to be confirmed as does the relationship of the pole concerned to the last cell division septum.

Bacterial cells are most sensitive to penicillin at the septation stage of the cell division cycle (21). The relative insensitivity to penicillin of the aseptate filaments compared with rods of the mutant is consistent with this fact. In E. coli it has been shown that the glycosidase, which is probably concerned with cell wall elongation is less sensitive to penicillin than the endopeptidase, which is probably concerned with crosswall formation (8).

D-Cycloserine (DCS) was the selective agent used in the isolation of this mutant (10). Penicillin was not used because many Bacillus species have inducible or constitutive penicillinases. The fact that growth of the filaments at 39 C and of the mutant at 26 C, which occur at similar rates, were totally inhibited by the concentration used for mutant selection shows that filaments are not more resistant than rods to DCS as long as their rates of growth are similar. However, since the mutant grows slower than

the parent at the restrictive temperature, DCS probably exerted its selective action by being less active against slower growing mutant cells than against faster growing parent cells. This sort of selection is reported to occur with penicillin (32).

The equal sensitivity of growth of rods and filaments of the mutant to rifamycin (1-50 μg/ml) shows that the mutation involved does not confer ts-rifamycin resistance. Thus, filamentation does not appear to be due to an RNA polymerase mutation. In Bacillus species, mutants resistant to 10 μg/ml of rifamycin have been obtained.

A preliminary experiment indicated that the division of filaments after temperature shift-down was sensitive to chloramphenicol, suggesting that aseptation at restrictive temperature does not involve reversible heat inactivation of a ts protein (1,23). This conclusion is supported by the fact that there is no sudden burst of division upon temperature shift-down of filaments (10).

In the temperature shift-up experiment, the proportion of spores formed could be used to measure the proportion of cells making sporulation septa if it was assumed that a constant proportion of cells forming septa completed sporulation. By comparing the time in the growth cycle at which shift-up prevented spore formation (Fig. 10) with the amount of DNA synthesized in the permissive culture at that time (Fig. 11) an approximate

estimate could be made of the time at the restrictive temperature required for the filamentation mutation to take effect. This time will be expressed in numbers of DNA replications. The shapes of the two growth curves in the shift-up and DNA experiments differ in the transition region from exponential phase to stationary phase. Therefore, the estimate has been made using both time of end of growth and culture turbidity. On the basis of time of end of growth, 10, 25 and 50% sporulation can occur after temperature shift-up when 1.5, 1.6 and 2.0 doublings of DNA could still occur. Thus, at least one but not more than two doublings of DNA must still be possible after temperature shift-up in order for sporulation to occur. It seems fair to conclude that after shift-up the round of DNA synthesis in progress is completed, the corresponding division septation occurs and a whole new round of DNA replication occurs which is then followed by sporulation septation. When shift-up is earlier in the exponential phase, two cell divisions and hence septations occur before the filamentous growth rate is established (10).

A decrease in the ability to sporulate after shift-up occurs when the temperature increase is carried out at the time of sporulation septation and engulfment (6,9). Possibly these stages are very sensitive to the 30 to 39 C shift because the extensive membrane synthesis and morphogenesis they involve requires a membrane whose

composition is exactly adapted to the existing temperature. The spores formed after the early temperature shifts (30 to 39 C) before the culture at 30 C is committed to sporulation, seem to be relatively heat sensitive. The reason for this is not known. Low frequencies of sporulation occurred after temperature shifts (increases) earlier in the growth cycle. This could be due to a combination of factors: exponential phase sporulation by the permissive culture, the leakiness of the ts TH14 mutation and sporulation by revertants.

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TABLE 1.--Radioisotope Ratios in SDS-PAGE Fractions of Membrane Protein Mixtures Made from Differentially Labeled Cells.

Source of mixture components[a]	Average Radioisotope Ratio			
	All fractions	Fraction A	Fraction Z	All fractions except A and Z
Mutant-39-^{14}C/mutant-30-^{3}H	0.289(77)[b]	0.463(3)	0.218(22)	0.309(52)
	0.881(77)	1.305(3)	0.674(22)	0.913(52)
Parent-39-^{3}H/parent-30-^{14}C	2.724(75)	2.890(3)	3.010(11)	2.665(61)
	1.811(75)	2.056(3)	1.985(11)	1.767(61)
Parent-39-^{3}H/mutant-39-^{14}C	1.261(80)	0.992(3)	1.615(14)	1.195(63)

[a]For example: mutant-39-^{14}C/mutant-30-^{3}H means the ^{14}C/^{3}H ratio of fractions of a mixture of membrane proteins derived from 39 and 30 C grown cells labeled respectively with ^{14}C and ^{3}H. The permissive temperature was 30 C and the restrictive temperature was 39 C.

[b]Numerals in parentheses indicate the number of gel slices containing the fraction. This number was used to calculate the average radioisotope ratio of the fraction.

TABLE 2.--Relative[a] Radioisotope Ratios of the A and Z Membrane Protein SDS-PAGE Fractions.

Source of mixture components[b]	Relative Radioisotope Ratio	
	Fraction A	Fraction Z
Mutant-39-^{14}C/mutant-30-^{3}H	1.498	0.705
	1.429	0.738
Parent-39-^{3}H/parent-30-^{14}C	1.084	1.129
	1.163	1.123
Parent-39-^{3}H/mutant-39-^{14}C	0.830	1.351

[a]Average radioisotope ratios of fractions A and Z were divided by the corresponding average ratio for all fractions except A and Z, which was obtained from Table 1. Dividing by the average ratio for all fractions, instead of the value for all fractions minus fractions A and Z, did not radically alter the above relative radioisotope ratios.

[b]See Table 1 for nomenclature key.

TABLE 3.--Variation of Relative[a] Amounts of the A and Z Membrane Protein SDS-PAGE Fractions with Strain and Growth Temperature.

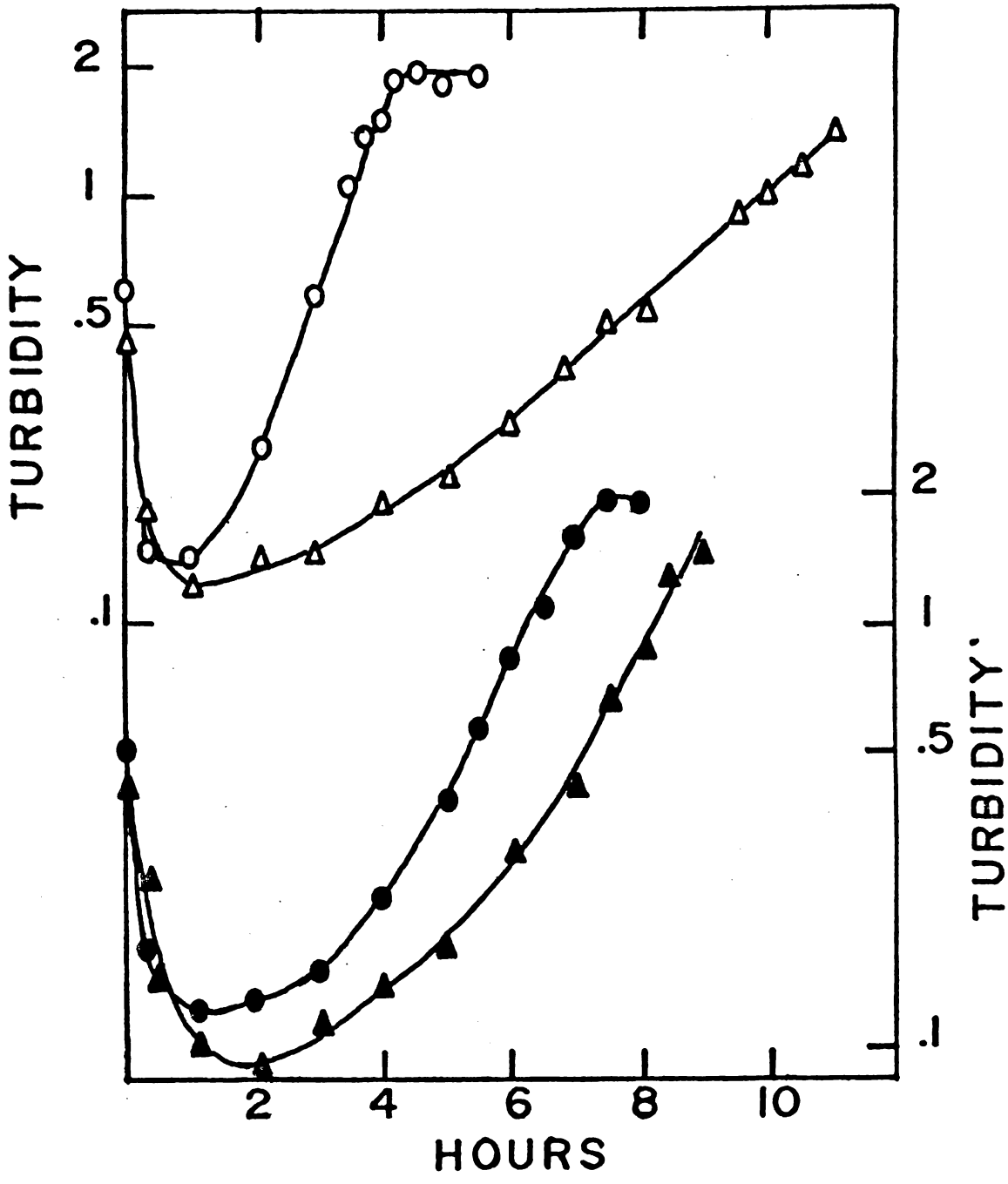
Fraction	Relative amounts of membrane protein fraction			
	Mutant strain		Parent strain	
	Restrictive[b]	Permissive	Restrictive	Permissive
A(80,000)[c]	1.46	1.00	1.22	1.08
Z(11,700)	0.72	1.00	0.98	0.87

[a]Relative amounts are based on a value of unity for the mutant grown at permissive temperature and were calculated from the relative radioisotope ratios in Table 2.

[b]Restrictive and permissive conditions were 39 and 30 C respectively.

[c]Values in parentheses indicate the approximate molecular weights corresponding to the fractions.

FIGURE 1. Germination and growth of parent strain and ts mutant TH14 strain spores. Symbols: 0, parent at 39 C; ●, parent at 30 C; Δ, TH14 at 39 C (filaments); ▲, TH14 at 30 C. Cultures behaving like these were used for cytoplasmic membrane preparation (see MATERIALS AND METHODS). Turbidity was measured at 600 nm.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TURBIDITY'

FIGURE 2. Densitometric scan of gels with membrane protein bands separated by SDS-PAGE. Migration of the bands was from left (-) to right (+). Symbols for sources of membranes: 39, restrictive temperature; 30, permissive temperature; TH14, mutant strain; parent, parent strain; A and Z are the protein fractions referred to in the text. The gels for the lower two scans (parent) were not electrophoresed as long as those for the upper two scans (TH14). Optical density is in arbitrary units.

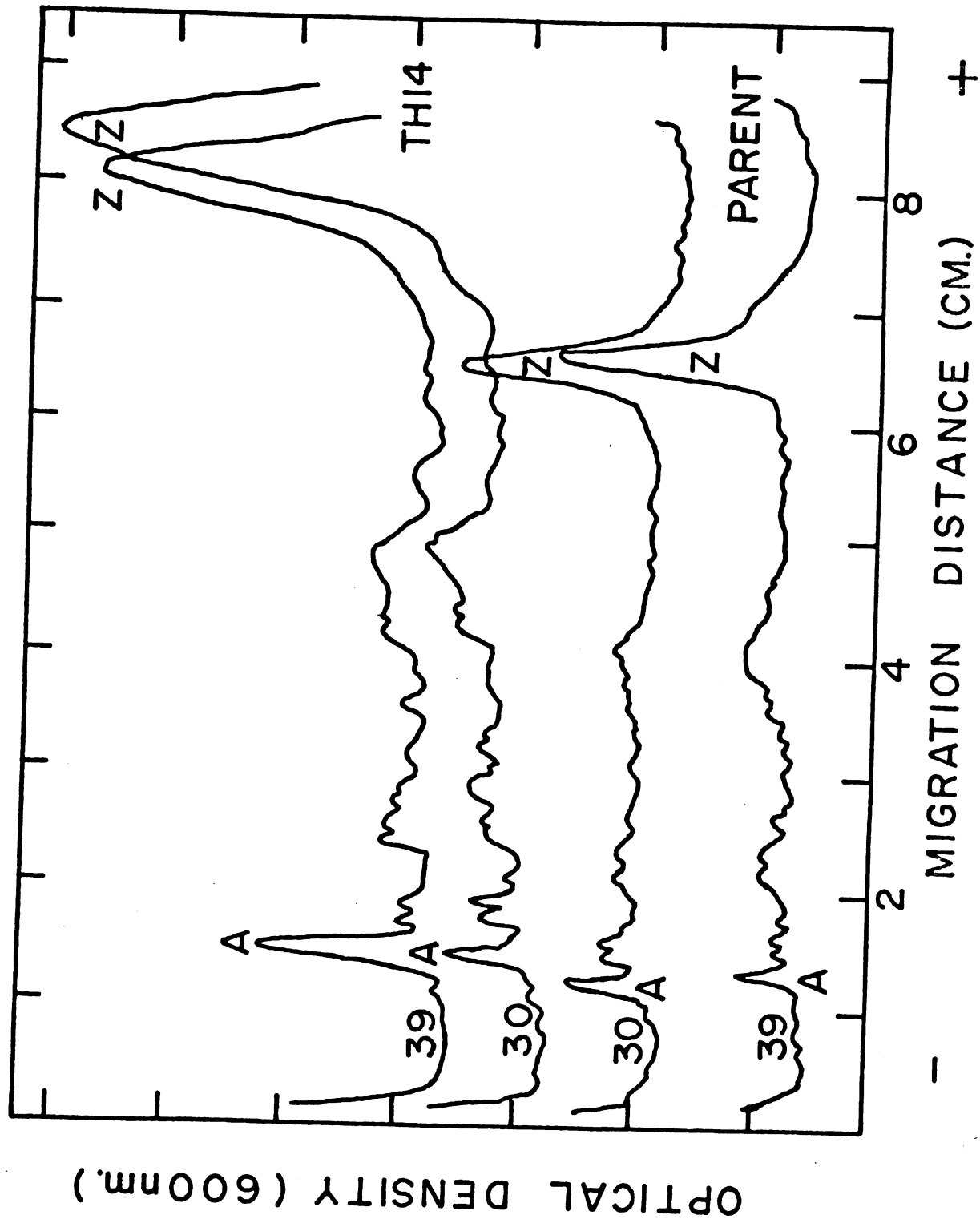

OPTICAL DENSITY (600nm.)
MIGRATION DISTANCE (CM.)
-
+
2
4
6
8
TH14
PARENT
Z
Z
Z
Z
A
A
A
A
39
30
30
39

FIGURE 3. Radiolabeled membrane protein SDS-PAGE profiles of ts mutant TH14. Symbols: continuous line, profile of tagged (L-leucine-4,5-^{3}H) proteins from permissively grown (30 C) cell membranes; dotted line, profile of tagged (L-leucine-U-^{14}C) proteins from restrictively (39 C) grown filament membranes; A and Z protein fractions are defined in the text. SDS-protein complexes migrated from left (-) to right (+).

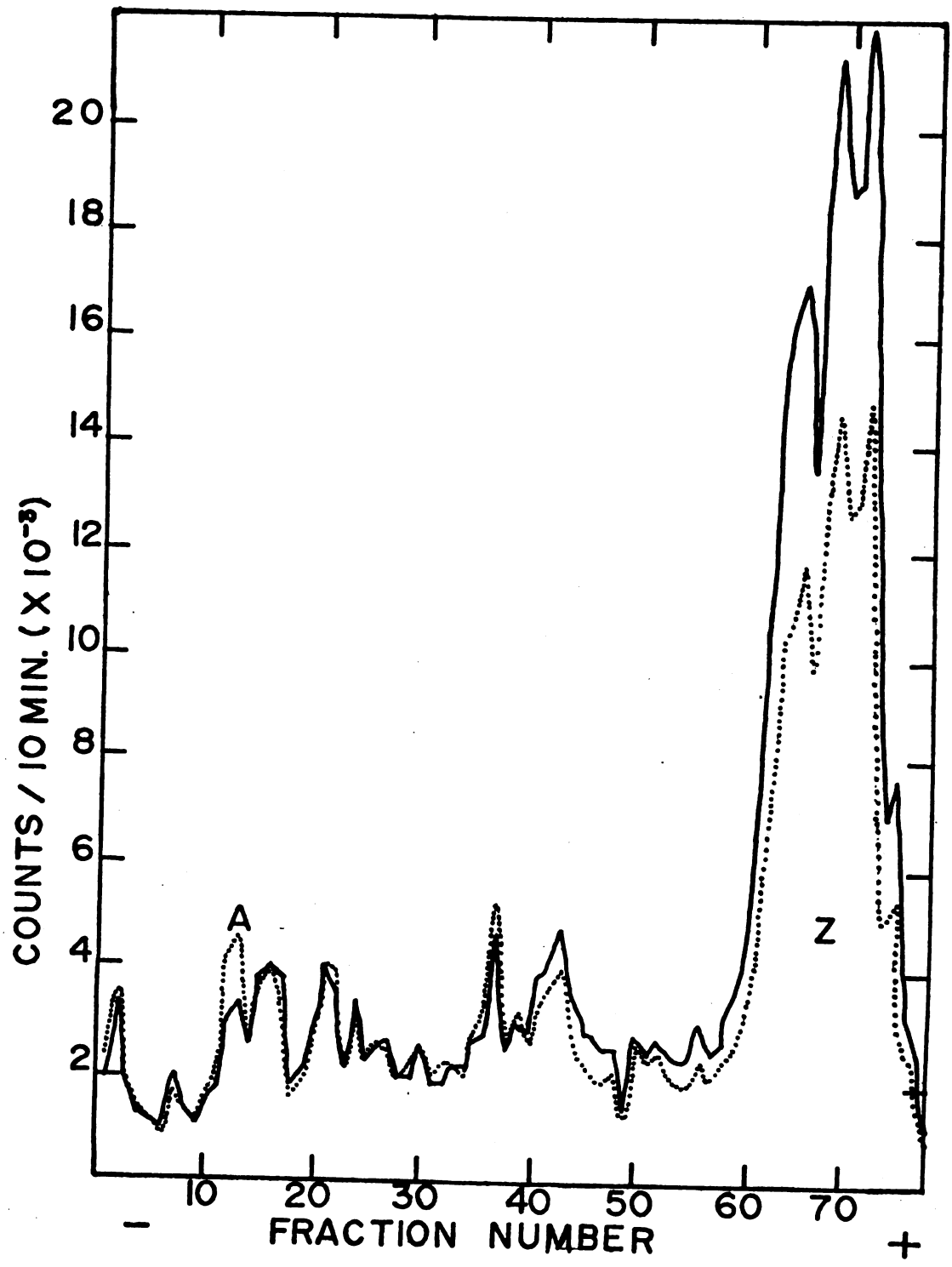

COUNTS / 10 MIN. (X 10⁻³)
20
18
16
14
12
10
8
6
4
2
A
Z
10
20
30
40
50
60
70
−
FRACTION NUMBER
+

FIGURE 4. Radiolabeled membrane protein SDS-PAGE profiles of ts mutant TH14. Symbols and other legend as in Figure 3. Unlike Figure 3 the profile of the L-leucine-U-^{14}C tagged restrictive proteins has been normalized relative to the profile of the L-leucine-4,5-^{3}H tagged permissive proteins. Normalization involves multiplying each ^{14}C value by the average radioisotope ratio for all gel slice fractions (see Table 1).

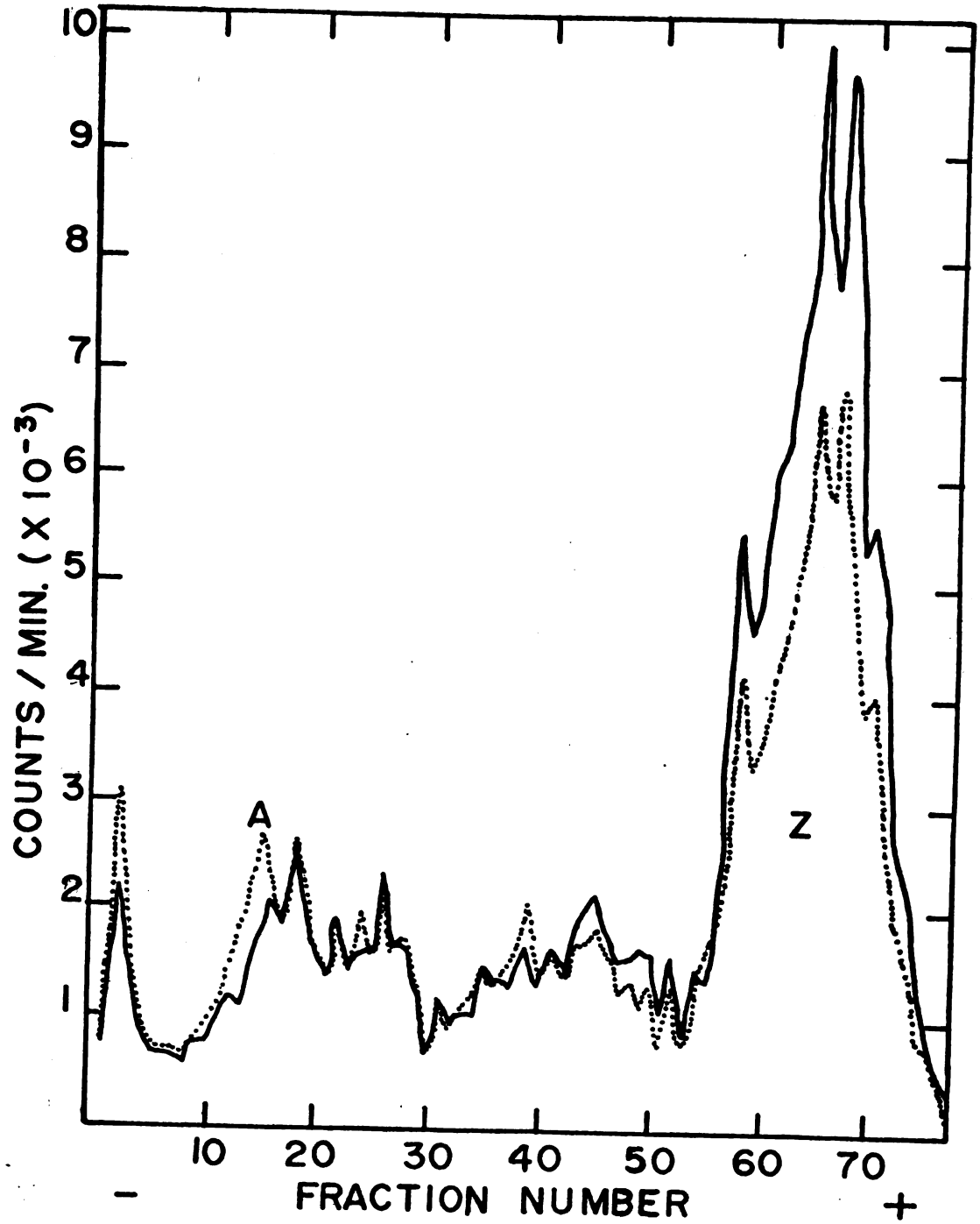

COUNTS / MIN. (X 10⁻³)
10
9
8
7
6
5
4
3
2
1
A
Z
10
20
30
40
50
60
70
FRACTION NUMBER
−
+

FIGURE 5. Radiolabeled membrane protein SDS-PAGE profiles of parent strain. Symbols: dotted line, profile of tagged (L-leucine-U-^{14}C) proteins from cells grown at 30 C; continuous line, profile of tagged (L-leucine-4,5-^{3}H) proteins from cells grown at 39 C; A, Z, + and - are as in Figure 3 legend. The profile of the L-leucine-U-^{14}C tagged proteins has been normalized as described in the Figure 4 legend.

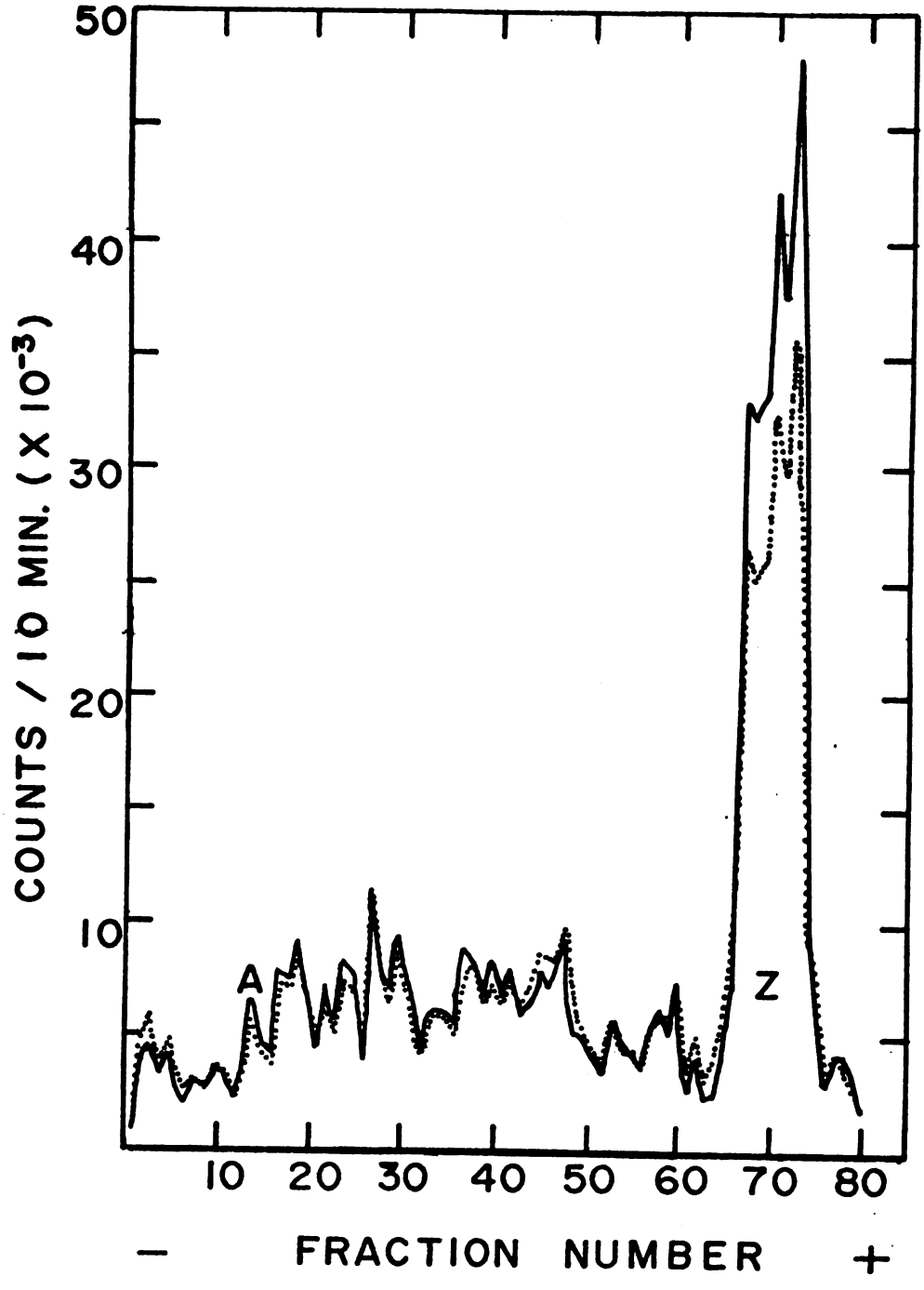

50
40
30
20
10
COUNTS / 10 MIN. (X 10⁻³)
A
Z
10 20 30 40 50 60 70 80
— FRACTION NUMBER +

FIGURE 6. Radiolabeled membrane protein SDS-PAGE profiles of a mixture of parent and ts mutant TH14 membrane proteins. Symbols: continuous line, profile of tagged (L-leucine-4,5-^{3}H) proteins from membranes of parent cells grown at 39 C; dotted line, profile of tagged (L-leucine-U-^{14}C) proteins from membranes of mutant filaments grown at 39 C; A, Z, + and - are as in the Figure 3 legend.

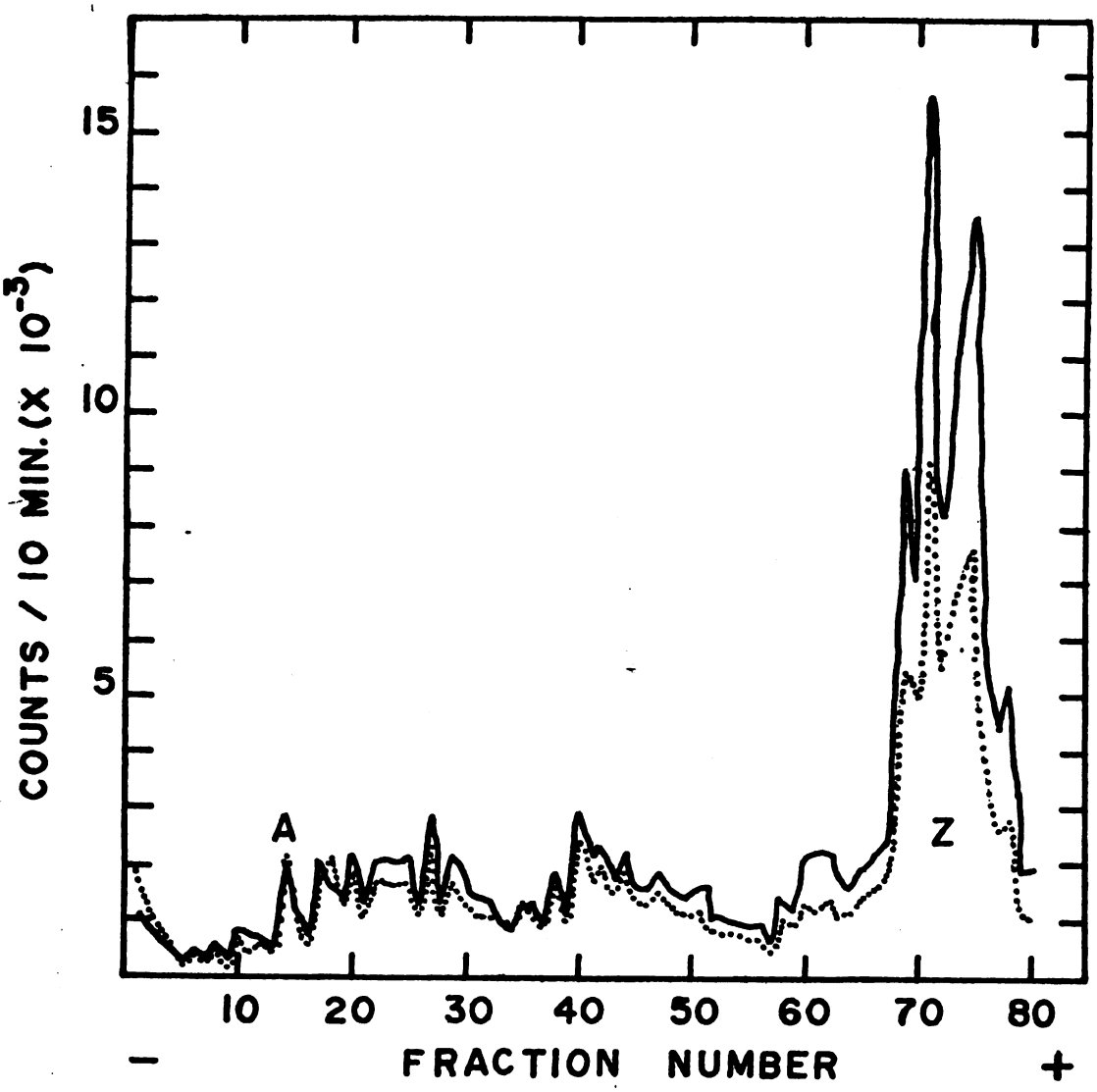

COUNTS / 10 MIN. (X 10⁻³)
15
10
5
A
Z
10
20
30
40
50
60
70
80
−
FRACTION NUMBER
+

FIGURE 7. Inclusion bodies in filament of ts mutant TH14. Symbols: I, inclusion bodies; D, DNA. The scale bar represents 1000 nm. Longitudinal and transverse sections of filaments 5 hr into the stationary phase are shown. The mutant and parent strains form short chains of cells. Inclusions are especially concentrated at filament poles.

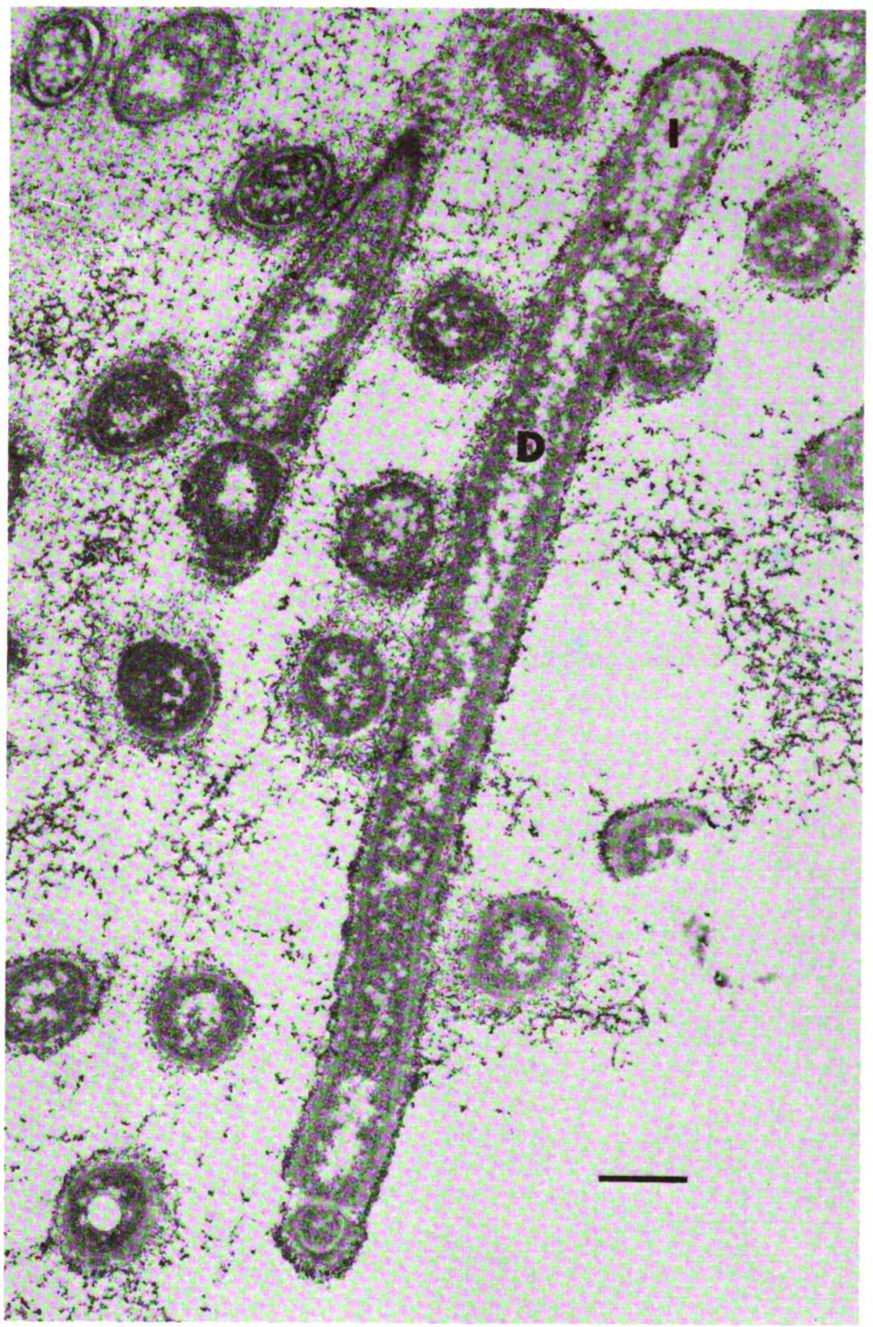

I
D

FIGURE 8. Inclusion bodies at pole of a filament of ts mutant TH14. Symbols as in Figure 7. The inclusions are not completely amorphous but substructure is not easily resolvable. There appears to be some capsular material. The scale bar represents 100 nm.

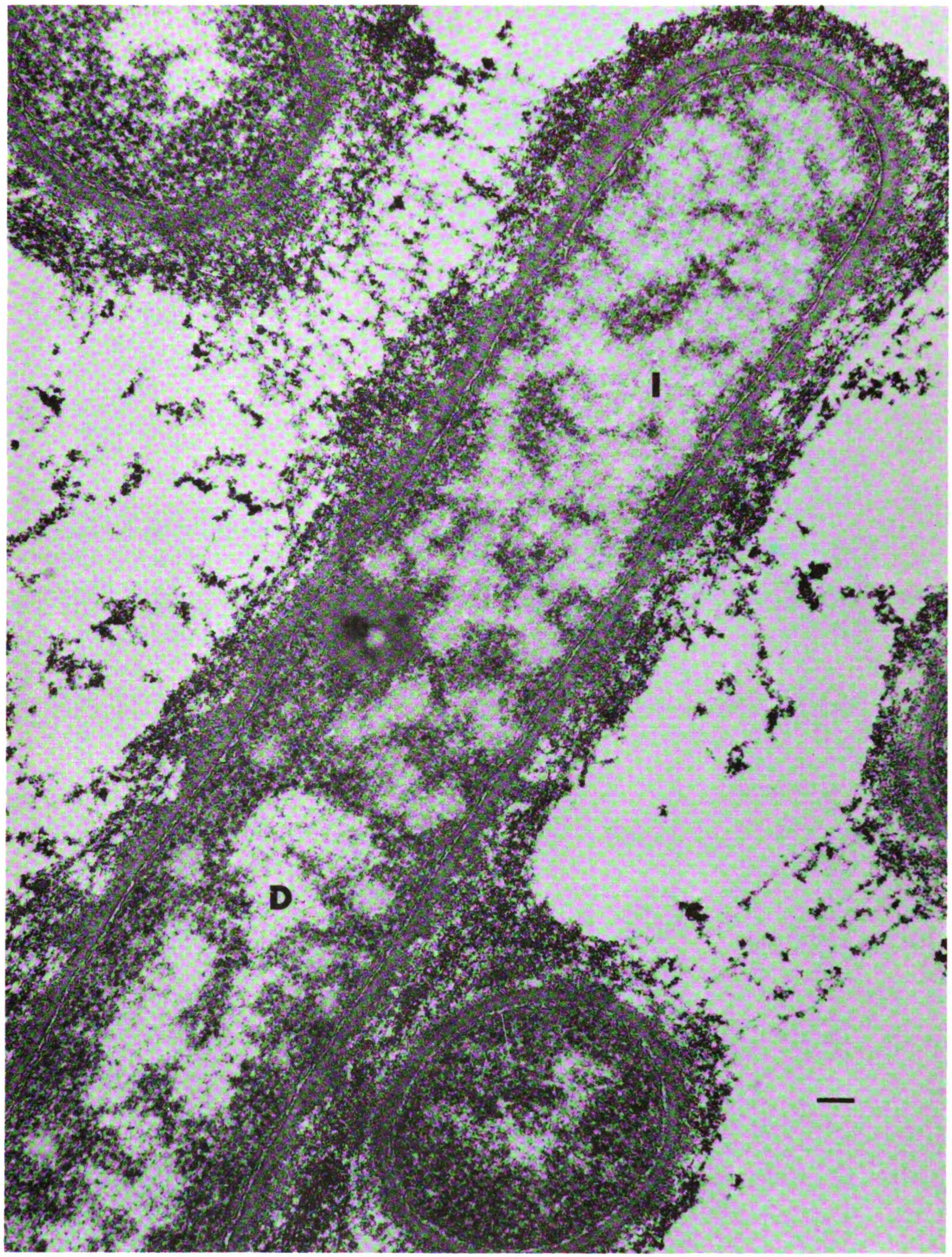

I
D

FIGURE 9. Effect of penicillin on growth of rods and filaments of ts mutant TH14. Growth at 26 and 39 C was measured turbidometrically at a wavelength of 600 nm. Benzylpenicillin (sodium salt) was used. Symbols: X, control and 1 unit/ml; 0, 10 units/ml; Δ, 100 units/ml; ▲, control; ●, 1, 10 and 100 units/ml.

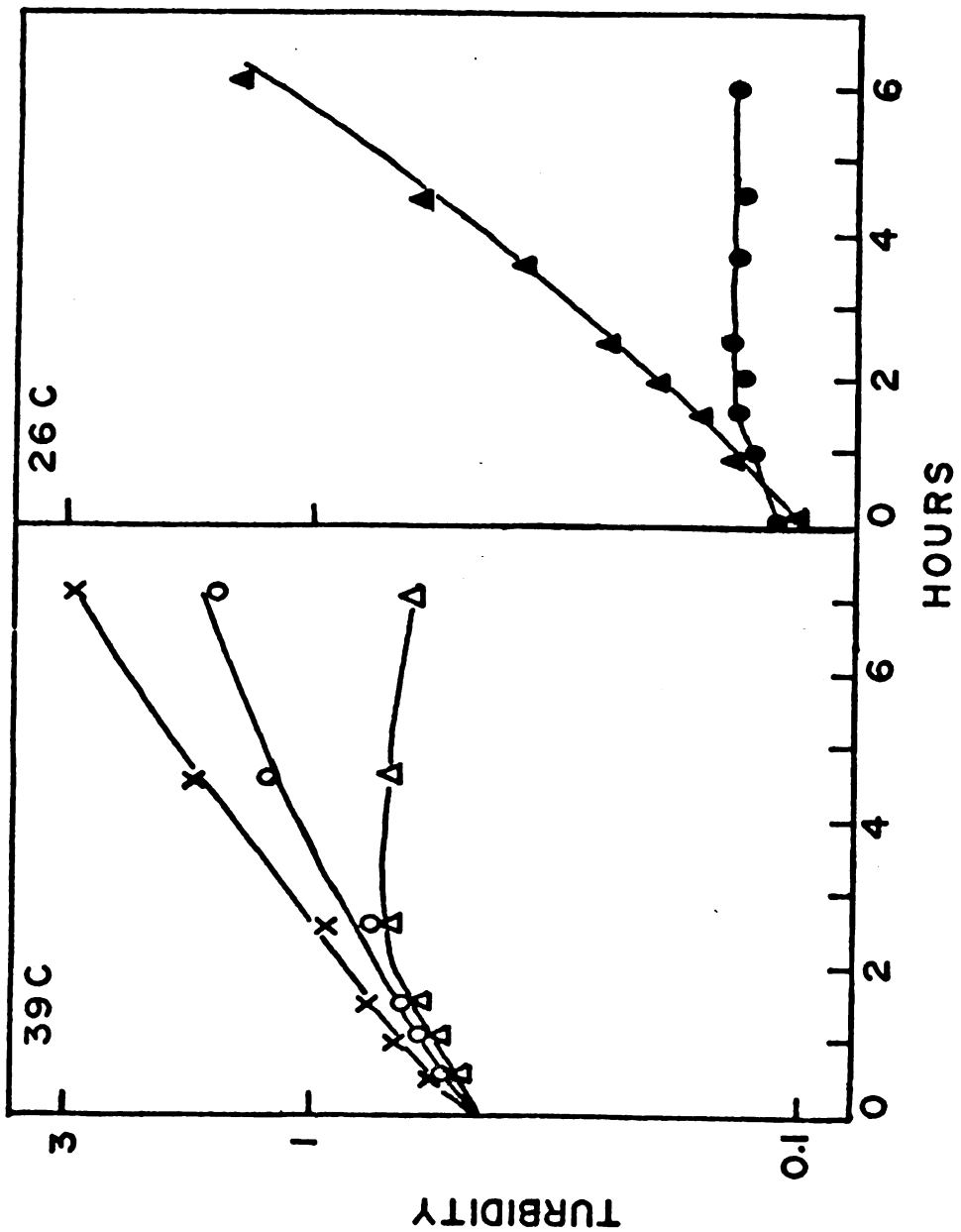

26 C
39C
TURBIDITY
HOURS
3
1
0.1
0
2
4
6

FIGURE 10. Effect of temperature shift-up on sporulation of ts mutant TH14. Samples (10 ml) of a permissively growing culture at 30 C were shifted to 39 C. Symbols: O, percent sporangia; Δ, percent heat resistant (70 C for 30 min) colony-forming units (cfu); ●, turbidity at 600 nm. Heat resistant cfu count normalized relative to the percent sporangia determination by arbitrarily assuming the maximum heat resistant cfu count corresponds to 1×10^8 cfu/ml. For percent sporangia determinations, 500 cells were counted at 0, 1, 2 and 3 hr (no spores were seen at these times) while 200 cells were counted at all other times. Sporangial determinations include sporangia with nonrefractile spores and free spores when present. The permissive culture at 30 C formed 74% sporangia compared with 73% at 39 C in the 13 hr shift-up sample.

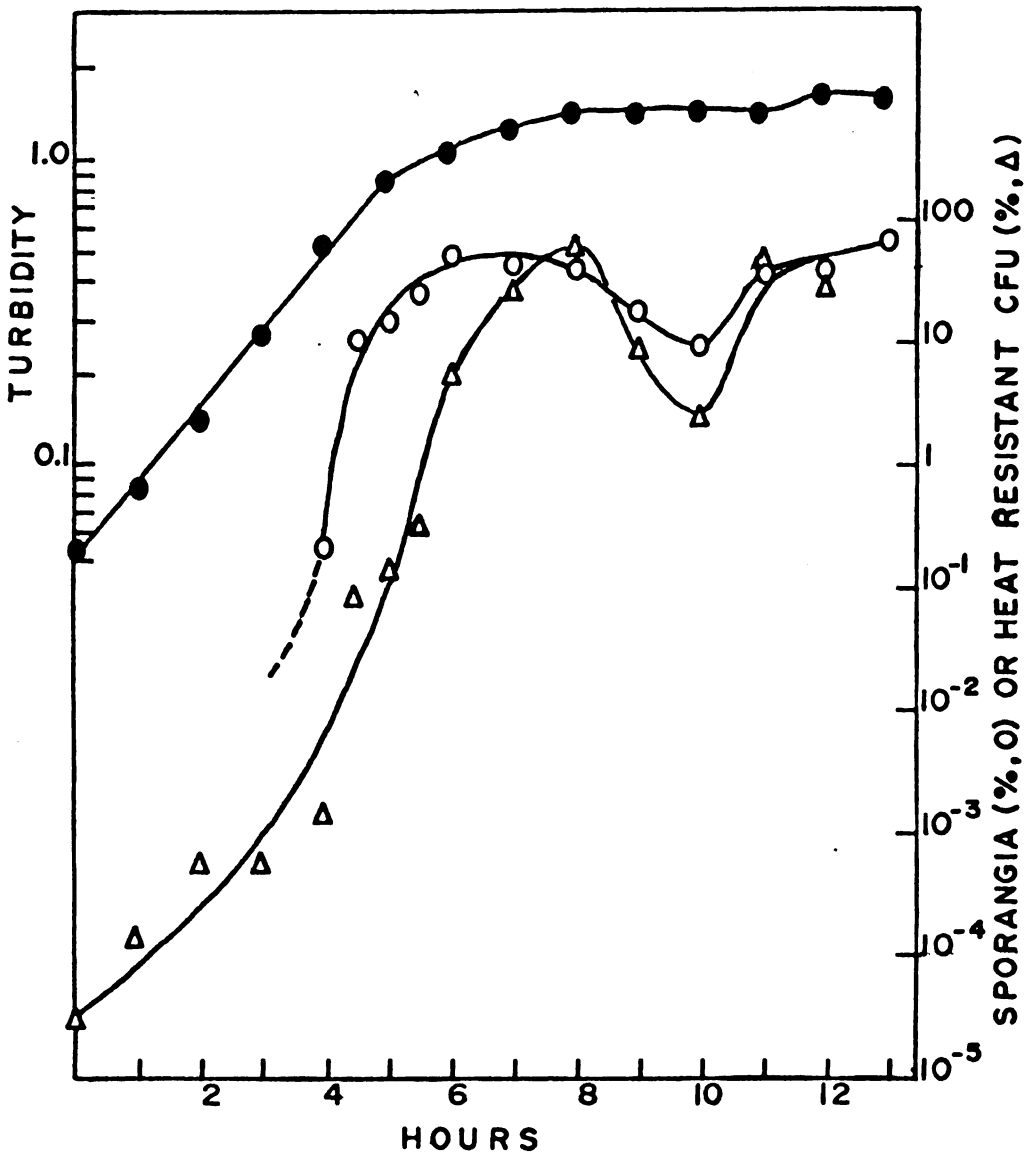
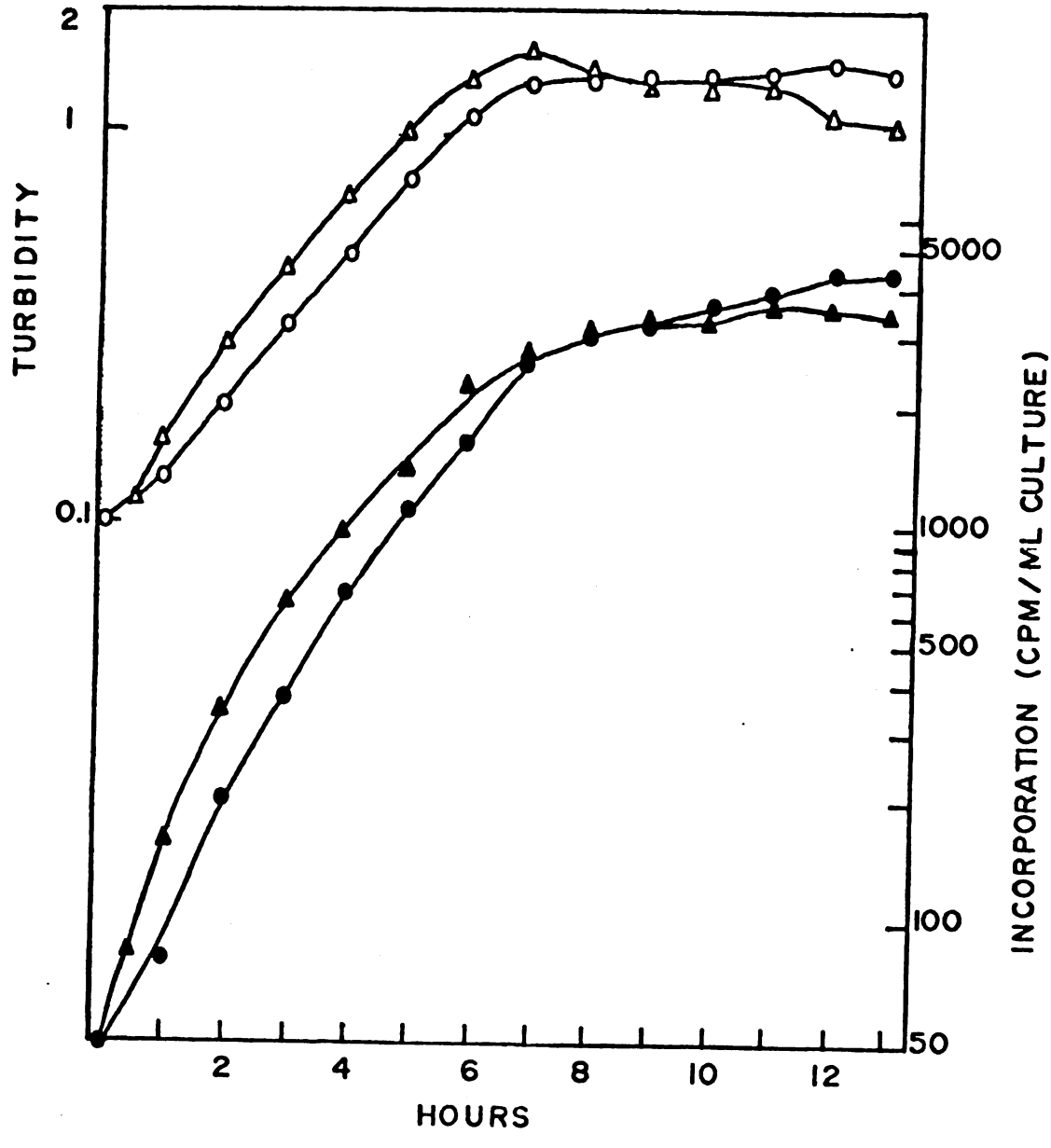

TURBIDITY
1.0
0.1
100
10
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
SPORANGIA (%,O) OR HEAT RESISTANT CFU (%,Δ)
2
4
6
8
10
12
HOURS

FIGURE 11. Growth and DNA synthesis of ts mutant TH14. Symbols: 0, culture turbidity at 26 C (permissive temperature); Δ, culture at 39 C (restrictive temperature); ●, DNA synthesis at 26 C; Δ, DNA synthesis at 39 C. Growth monitored by measuring culture turbidity at 600 nm. DNA content of the cultures measured as uracil-2-^{14}C incorporation (cpm/ml culture) into the alkali-stable fractions of cells as described previously (10). Prewarmed (26 C) culture medium (200 ml) containing uracil (200 µg/ml) was inoculated with actively growing cells from an overnight culture grown in the same conditions. Uracil-2-^{14}C (20 µCi total) was added and after 30 min at 26 C the culture was split into two halves. One-half was kept at 26 C and the other shifted to 39 C.


2
1
0.1
TURBIDITY
5000
1000
500
100
50
INCORPORATION (CPM/ML CULTURE)
2
4
6
8
10
12
HOURS

CONCLUSION

Results of studies of the properties of a thermosensitive filament forming mutant of Bacillus megaterium have been presented. These suggest that the mutation has a common effect on cell division septation and sporulation septation. This emphasizes the predicted relationship between these two processes.

How does the mutation exert its effect on septation? This question cannot be answered yet. However, there are several noteable properties of the mutant that should be considered. Gross DNA synthesis and segregation appear to be unaffected by the mutation. The aseptation effect of the mutation is not apparent until after a time-delay equivalent to 1 to 2 rounds of DNA replication, i.e. 2 division septations. There is also a delay in recovery from the effect of the mutation although this phenomenon has not been analyzed in terms of cell division.

The mutation caused a decrease in exponential growth rate at restrictive temperature compared with the parent strain and this effect became apparent before the filamentation effect. Inclusion bodies of undetermined composition accumulated in the mutant by the time filamentation started. The mutation caused quantitative changes in at least 2 membrane proteins.

One hypothesis of the mode of action of the ts mutation is that a metabolic process is altered which causes a reduction in growth rate at the restrictive temperature relative to the parent strain. The limitation leads to accumulation of the inclusion bodies and to aseptation. An alternative hypothesis would be that the control process for septation is upset by the mutation. This results in partial growth inhibition and, when septation signals stop, filaments are formed. Materials needed for septation then accumulate. Obviously, a study of the nature of the inclusions will be critical to understanding the mode of action of the mutation.

APPENDIX

Bacterial Sporulation as a Modified Procaryotic Cell Division

By

A. D. Hitchins and R. A. Slepecky

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*This review was partially prepared at the Department of Microbiology and Public Health of Michigan State University while one of the authors (A. D. H.) was a student there.

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Bacterial Sporulation as a Modified Procaryotic Cell Division

by

A. D. HITCHINS*
R. A. SLEPECKY

Biological Research Laboratories,
Syracuse University,
New York 13210

This article reviews the idea that the bacterial spore is a small cell, formed by an asymmetric cell division and enveloped by the larger sister cell. Both processes seem to be morphological expressions of unbalanced growth resulting from shift down growth conditions.

SEVERAL authors[1-9], observing that both bacterial sporulation and cell division involve compartmentalization of a cell by means of septum formation, have stated that sporulation is a modified or atypical cell division. Because this idea has been mentioned rarely in reviews concerning sporulation[1,2,10-16], and only briefly in the context of sporulation synchrony[17], we present here an evaluation of a hypothesis which explains sporulation in terms of the more general phenomenon of cell division. This hypothesis suggests that sporulation is a modified cell division and an expression of unbalanced growth resulting from shift down growth condition. It gives a logical framework on which to arrange much of what is known about sporulation.

Table 1. HYPOTHETICAL RELATIONSHIP OF SPORULATION AND CELL DIVISION

Stage	Sporulation event	Proposed relationship to cell division
I	Preseptation: DNA in axial filament form	Slow separation of DNA replicated in late log and early stationary phases
II	Septation	Asymmetric cell division results in two unequal sized cells each with a part of the DNA (mother cell and smaller germ cell)
III	Envelopment	Membrane synthesis out of step with cell wall formation (stage IV). Envelopment begins encystment of germ cell
IV	Cortex and germ cell wall formation. Dipicolinic acid (DPA) made	Peptidoglycan synthesis; equivalent to cell wall formation. DPA is a product of secondary metabolism
V	Coat formation	Coat and cortex encyst the germ cell. Coat protein a result of secondary metabolism
VI	Maturation of spore	Net result of asymmetric cell division is one germ cell encysted in products of the mother cell
VII	Lysis of mother cell and release of free spore	Lysis as a result of lack of further division

Sporulation requires an orderly sequence of seven well defined morphological steps for its completion. Table 1 compares the morphological stages of sporulation as currently described[16], with sporulation interpreted as a modified cell division. Sporulation seems to be the resultant of two factors. Factor one is the innate ability of spore forming cells to complete a round of DNA replication and to partition the replicated molecules by means of a septum as late as 2–3 h after the end of growth. In essence this means cell division without net growth. The second factor is the effect of change of metabolic pattern, resulting from exhaustion of growth limiting substrate, on this cell division. The metabolism of the cells changes from that typical of logarithmic growth, through a shift down growth pattern, to a pattern of endogenous metabolism which includes turnover and consequent reallocation of cellular materials. This results in unbalanced synthesis; there is an accumulation of new end products (secondary metabolism) and, most importantly, chemical modifications and changes in the relative timing of synthesis of components common to the vegetative and sporulating cells.

Perhaps such metabolic imbalance exerts its first effects on the DNA of the sporulating (dividing) cell. The first visible effect of the change, however, is the marked size inequality of the resulting daughter cells (asymmetric division). This differential effect on the daughter cells becomes progressively amplified so that the later stages of sporulation bear little apparent relationship to cell division. These stages include envelopment of the small cell by the large one and encystment of the small cell in products of the large cell. Finally, the large cell lyses, freeing the encysted small cell.

During the first stage of sporulation, the DNA assumes a linear configuration along the long axis of the future sporangium (axial filament stage). This has usually been considered unique to sporulating cells, but similar configurations have been seen during normal cell division[13,16] and in a non-spore-forming organism[19]. Several facts suggest that axial filaments could be formed as a result of slow separation of newly replicated DNA molecules[13,16]. First, the last round of DNA replication, which presumably starts in the late log phase of growth, continues during the axial filament stage[20]. Second, the spore germ cell receives half of the DNA originally present in the sporangium[10,11]. Thus stage I can be regarded as the completion of the DNA replication stage of cell division. How spores with a reported chromosome content of two[21,22] fit into this interpretation is not clear.

* Present address: Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823.

Formation of Septum

As in cell division, compartmentalization of the duplicated genome of the sporangium occurs during stage II when a septum is formed due to membrane invagination. At the fine structure level, sporulation septation seems to be similar to cell division septation[8,18,33], but differs in that no cell wall material is formed between the two membranes of the septum. But when sporulation is upset, as in rejuvenation experiments[34] or in certain mutant strains[18,35], cell wall material may be deposited. It should be noted that there is some recent morphological evidence for the presence of cell wall material between forespore septa in clostridia[36]. In addition there is chemical evidence for peptidoglycan formation[37] during preseptation.

This is probably due to turnover which is known to occur in non-growing bacteria in some conditions[38], but net cell wall synthesis may occur in *Clostridium pectinovorum* which increases markedly in size during preseptation[1,6]. Much more precise correlation of the timing of peptidoglycan synthesis with that of septation has been demonstrated by the detection of significant incorporation of diaminopimelic acid during septation (unpublished results of R. Greene and R. A. S.) in pulse labelling experiments during synchronous sporulation[17]. These data indicate that some rearrangement of cell wall material, if not complete resynthesis, is necessary for septum initiation. Whether the presence of a cell wall is necessary for septation is not clear, however, even though work with sporangial protoplasts has shown that subsequent envelopment (stage III) requires the presence of an intact cell wall[39].

Antibiotics known to inhibit cell division by inhibiting cell wall synthesis also inhibit sporulation septation[30]. This fact confirms the involvement of peptidoglycan in septation initiation but, more importantly, highlights the similarity of the sporulation and division septation processes[30]. This similarity is further accentuated by the fact that phenethylalcohol (PEA) inhibits both cell division and sporulation septation[17,21] although the two processes differ in sensitivity. This effect of PEA is especially noteworthy because the compound is believed to affect the site(s) controlling DNA replication and cell division[21].

Aseptate[13] and multiseptate[18,33] sporulation mutants and the peptidoglycan-less septa of wild type strains pose a problem for the hypothesis. If sporulation septation and division are related, how can sporulation septation be prevented or altered by mutations that do not also affect cell division septation ? One answer is that such mutations are conditionally expressed. A possibly analogous situation exists in the abnormal cell division septation of *Bacillus* mutants[34]. These are conditional mutants but unfortunately the effect of the mutations on sporulation septation was not reported. Conditional sporulation septation mutants might, however, be expected to be more frequently isolated than non-conditional mutants according to the hypothesis, for routine methods for selecting sporulation mutants depend on the mutant being able to form a population[13] by normal cell division.

Possibly the most important difference between cell division septation and sporulation septation is the asymmetric positioning of the sporulation septum very near to one of the sporangial poles. It must be noted, however, that asymmetric septation is not specific to bacteria which produce spores. It occurs in minicell formation[35], division of filaments[36], division of a blue-green alga[37] and in yeasts[38]. The asymmetry of the sporulation septation can probably be regarded as the first morphological expression of unbalanced growth resulting from the establishment of shift down growth conditions[39] during stage I[4,40,41]. Sporulating cells have a physiology similar to cells growing on acetate[15] in that during stage I sporulating cells adapt to by-products of growth, acetate and pyruvate[10]. As these by-products are utilized, endogenous metabolism (as evidenced by macromolecular turnover)[10,11,14-16] begins to dominate sporulation physiology. The gradual slowing of growth to the zero or near zero rate characteristic of the stationary phase, and the cell's increasing dependence on endogenous metabolism, could lead to unbalanced synthesis. This could then result in an asymmetric division as suggested for certain yeasts[38]. Microcycle sporulation[41] illustrates the effect that the condition of the medium can have on outgrowing spores in determining the choice between division or sporulation septation. If sporulation septation is essentially a modified cell division, then it is a division that can occur in conditions of virtually no net growth. Such relative independence of growth and cell division is sometimes apparent in vegetative cells[42].

One can only speculate as to the nature of the underlying macromolecular events which lead to the asymmetric septation. Presumably a relationship between membrane formation and DNA replication is involved. In conditions that limit growth, one of the daughter DNA molecules of the last replication may be less active in the control of synthesis. The dominant DNA molecule might affect the positioning and availability of membrane attachment sites[17] for the other DNA molecule as suggested in the site-territory model[44] for the control of DNA replication. Attachment sites seem to be more important than the actual DNA molecules, because which of the two daughter DNA molecules—the "old" or the "new"—enters the future germ cell of the spore is randomly determined[45] in *B. cereus* just as is the segregation of daughter DNA molecules in *B. subtilis* cell division[46]. But the latter report is contradicted by a previous finding[47] and the recently reported demonstration of DNA excretion (about 40 per cent) during *B. subtilis* sporulation[21] suggests that daughter DNA molecule segregation patterns may be more difficult to determine than previously thought.

Germ Cell Genome

In addition, because the germ cell is several times smaller in volume than the sporangial cell, this may necessitate a different supercoiling of the DNA molecule, which in turn could affect the functioning of the germ cell DNA. It has been suggested on the basis of photochemical data that spore DNA is less hydrated than vegetative DNA[48] and that the configuration is altered during germination[49] of the spore to reform a vegetative cell. It is not clear at what stage of sporulation the spore's new DNA configuration would be established.

The apparent inactivity of the future spore or germ cell genome seems to be only temporary or partial. Thus the newly formed germ cell increases in size[50] presumably as a result of net synthesis. Protein synthesis is known to occur in the germ cell compartment after septation[51] but whether this is net synthesis is not clear, for the amount of protein degradation occurring there is unknown[51]. An interesting suggestion is that the engulfed germ cell is in a step-up growth condition, perhaps caused by amino-acids formed by the engulfing cell (H. L. Sadoff, personal communication). The location of the cortical membrane or germ cell wall suggests that it is synthesized by the germ cell. The larger size of the mother cell coupled with its probable synthesis of the coat layers, cortex and its membrane envelopment of the germ cell, however, suggests that it synthesizes more actively than the germ cell. Studies on the relative types and amounts of metabolism by the germ and mother cells are hampered by the technical difficulty of separating the two cells[11]. Germ cells have been isolated some time after engulfment, however (D. Karp, D. Lang and D. G. Lundgren, personal communication), and this system should help to clarify some of the preceding problems.

Sporulation further resembles cell division when the two compartments resulting from sporulation septation behave like normal cells; they can both divide in certain conditions. Thus the mother cell will divide when rejuvenated[34,41]. There are positive[34] and negative[41] reports

of germ cell rejuvenation just after septation but before its envelopment by the mother cell. The germ cell of the mature spore can either divide or resporulate without normal division after germination[48]. The two compartments also resemble cells in that each can form a protoplast if the sporangial wall is removed just after septation but before engulfment[5,29,52]. Association of mesosomes with septation in sporulation and cell division[7] suggests that the septation processes are related. The changes in mesosome structure during growth and sporulation are also very similar[53], but the role of mesosomes and membranes in DNA replication and distribution between daughter cells is not clear[54].

Events subsequent to septation can be interpreted as further expressions of the mother cell's unbalanced synthesis as first manifested by the asymmetric septation. Envelopment (stage III) of the germ cell protoplast by the mother cell protoplast can be regarded as the consequence of mother cell membrane synthesis being out of step with net cell wall synthesis, which is delayed until stage IV (cortex formation); membrane synthesis within the confines of the sporangial wall inevitably leads to engulfment. This tends to be supported by the fact that, after treatment with lysozyme, the germ cell protoplast is not enveloped by the mother cell protoplast even though still attached to it[29]. No data are available on the timing of membrane synthesis in relation to septation and envelopment although very active incorporation of labelled phosphate into phospholipid during stage I has been reported[55]. The use of Nomarski interference contrast optics enables easy quantitation of *Bacillus megaterium* septation and envelopment[56], and so it should now be possible to correlate more precisely biochemical events, such as phospholipid synthesis, with the earliest morphological events of sporulation.

Germ Cell Wall

Cortex formation (stage IV) involves peptidoglycan formation, and it has been suggested that this is equivalent to cell wall formation by the mother cell[4]. The germ cell wall (cortical membrane) peptidoglycan appears before the cortex in *B. subtilis*[18]. The delay in the synthesis of cortex is presumably a consequence of a continuation of unbalanced synthesis in the mother cell.

The spore coat is believed to be synthesized by the mother cell[10,12,51], for it is located there. As a result, some mother cell cytoplasm lying between the coat and the envelopment membrane is incorporated into the maturing spore[10]. The fate of the envelopment membrane is not known. Continuation of unbalanced growth leads to lytic disruption of the mother cell, but the germ cell, being in a rejuvenated state and surrounded by a resistant coat, survives. Coat and cortex formation can be regarded as an encystment of the germ cell by the mother cell[7]. Thus the mature spore consists of a small cell (the germ cell) with its cell wall surrounded by products of unbalanced growth of a second cell (the mother cell).

Many of the molecules involved in sporulation are similar to those involved in vegetative growth. These include proteins[11], lipids[11,57], DNA[10] and peptidoglycan[10,11,58]. This information is consistent with sporulation being a modified cell division. It implies that many of the cell's genes function in both sporulation and growth. These molecular differences reported for peptidoglycan[58], proteins[11] and DNA[59] seem to be minor modifications.

The morphological changes that occur during sporulation seem to be principally the result of changes in the relative timing and quantity of membrane and cell wall materials that are synthesized. In this way, different shapes may be made from similar materials. The membrane seems to play a particularly important part in shape changes, for it is likely that the cortex and coat shapes are determined by the membrane topography. The importance of unbalanced cell wall synthesis in shape changes has been reported in a non-sporulating organism[60] and the effect of partial or complete lack of cell wall on shape in L forms is well known[61].

On the other hand, some substances are sporulation-specific and their production probably involves, except perhaps in the case of the exosporium, expression of genes not concerned with vegetative growth. These substances[10,11,13] include the coat, dipicolinic acid (DPA), antibiotics, toxins, the exosporium, parasporal bodies, and certain enzymes. Most of these substances are probably synthesized by the mother cell except perhaps the DPA. The exosporium, parasporal body, toxins and possibly the antibiotics are not produced by all sporulating organisms. These sporulation specific substances can be regarded as products of secondary metabolism[62] consequent on the unbalanced growth conditions. Some genes expressed during vegetative growth are not expressed or are only slightly expressed during sporulation as evidenced by the reduced amounts or absence of certain enzymes in spores[11].

Both vegetative and sporulation genes are expressed during sporulation, and so the RNA should be similar in sporulating and vegetative cells except for the *m*RNA involved in the transcription of sporulation- or vegetative-specific genes. Available evidence[10,11] does not refute this. Ribosomal RNAs from spores and vegetative cells of *B. subtilis* are reported to be genetically similar[63].

We feel the hypothesis that sporulation is a modified cell division is acceptable for the following reasons. First, the spore contains an independent cell (the germ cell) which must have arisen by cell division because, in accordance with Virchow's postulate, a cell can only be formed by the division of a pre-existing cell. Second, the morphological and compositional evidence available is consistent with the hypothesis which emphasizes the similarities of spore and vegetative cell composition and structure as well as accommodating the differences. The hypothesis incorporates the modern concepts of unbalanced growth, metabolic shifts, turnover and secondary metabolism in attempting to explain the dynamics of sporulation. Some of the evidence, especially that regarding the earliest stage, is only fragmentary or inferential. Likewise the details of how unbalanced growth conditions later lead to unbalanced synthesis when there is no net growth remain obscure. Several possible mechanisms have been suggested[2,15,64] and will not be discussed here. The slowness of sporulation (6–8 h) and the similarity of the early stages to cell division, however, suggest that there is a gradual modification of the vegetative metabolic pattern, rather than a relatively sudden switch from a vegetative to a sporulation genome. Thus the sporulating cells become committed to each stage of sporulation as it is approached, rather than becoming committed to the whole process all at one time[65]. Most significantly, the hypothesis suggests new areas of investigation. One such key area is the precise relationship of asymmetric septation to cell division septation. Although understanding the asymmetric septation may have to await advances in our knowledge of cell division, it is conceivable that knowledge of sporulation septation may be useful in understanding cell division septation. Sporulation septation studies are not as dependent on synchrony, although this is possible[17], as cell division septation studies where there are overlapping cycles of septation in asynchronous cultures. Indeed, the hypothesis suggests that cell division itself, rather than sporulation, is the simplest example of bacterial differentiation at the cellular level of complexity. Other sporulation phenomena where the hypothesis may prove a useful guide to investigation include log phase sporulation[64] and oligosporogeny[13].

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