TEMPERATURE-SENSITIVE MUTANTS OF BACILLUS CEREUS T IMPAIRED IN ABILITY TO SPORULATE AND ALTERED IN RESISTANCE TO LYSIS BY BACTERIOPHAGE

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ABSTRACT

TEMPERATURE-SENSITIVE MUTANTS OF BACILLUS CEREUS T IMPAIRED IN ABILITY TO SPORULATE AND ALTERED IN RESISTANCE TO LYSIS BY BACTERIOPHAGE

By

Gerard Nicholas Stelma Jr.

The objectives of this study were to isolate temperature-sensitive mutants of Bacillus cerenus T blocked at an early stage of sporulation and to use these mutants to obtain information on the nature and number of functions which are indispensable to the sporulation process. Five temperature-sensitive mutants were isolated from cells treated with N-methyl-N-nitro-N-nitrosoguanidine. Preliminary investigations of these mutants indicated that each of them is susceptible to an agent which causes them to lyse in a sporodic manner. This lytic agent was identified as a bacteriophage carried by the parent strain in a latent form but induced at high frequencies in the mutants. Three of the mutants have lost superinfection immunity.

Observations of the phage under the electron microscope reveal that they are pleiomorphic. One morphological type has heads 65 to 70nm in diameter and

tails 200nm in length with triangular base plates. The second type has heads identical to those of the first, but the tails are variable in length; a variable number of plates occur at irregular intervals along the tails and perpendicular to the axis of the tails.

One of the mutants, JS22, becomes spontaneously cured of the phage genome at a high frequency. Clones are cured if they no longer produce plaque-forming phage and simultaneously become sensitive to the phage. Sedimentation of DNA from JS22 and from the parent strain in CsCl-ethidium bromide density gradients reveals that this spontaneous curing is not due to the occurrence of the phage genome as a plasmid. Strains cured of the lytic phage still carry an apparently defective phage which cannot produce plaques.

The lytic phage produced by the mutants was compared with the two B. cereus phages which it most closely resembled. Comparisons in size, chloroform sensitivity, host range, and antigenic components establish that this phage is unique, and hence it has been designated as lytic phage 22 (LP-22). LP-22 is not able to mediate generalized tranduction in B. cereus T.

The time course of phage release was measured in broth cultures of JS22 and the parent strain at both restrictive (37C) and permissive (26C) temperatures. Maximal phage titers in cultures of the mutant grown at

37C are $4x10^9$ plaque forming units per milliliter (pfu/ml), while those of the parent strain are 10^4 pfu/ml. Maximal phage titers in cultures of the mutant and parent strain grown at 26C are $7x10^8$ pfu/ml and 10^3 pfu/ml respectively. At both temperatures over 99% of the phage produced by the mutant and over 90% of those produced by the parent strain are released after the end of exponential growth. This late release of phage does not appear to be due to a requirement of the phage for a sporulation-specific RNA polymerase or a sporulation-specific protein synthesizing system; infected cells of JS29, a mutant which has lost its superinfection immunity, are able to produce high titers of phage during early exponential growth. The only condition in which induction of LP-22 has been observed is mutation to asporogeny. Ultraviolet light, mitomycin C, cis dichlorodiammineplatinum (II) and growth at elevated temperatures all fail to induce the phage in cultures of the parent strain.

JS22-c, a strain of JS22 which has been cured of the LP-22 genome, was investigated in some detail in order to determine the nature of the genetic lesion which prevents it from sporulating at 37C. Growth studies of the mutant and parent strain at 37C reveal that the vegetative growth rate of the mutant is identical to that of the parent strain. Some biochemical events associated with the onset of sporulation, such as production of

extracellular and intracellular proteases and production of alkaline phosphatase, occur normally at 37C. Failure of the pH to increase after the end of exponential growth in 37C cultures of JS22-c indicates that the mutant is unable to oxidize, via the tricarboxylic acid cycle, the organic acids which accumulate in the medium during growth. Examination by electron microscopy of cells of JS22-c incubated at 37C for the time period that allowed sporulation to occur in the parent strain has revealed that the cells are blocked at stage 0 of sporulation. Cultures of the mutant produce approximately 10^3 heat-stable spores per ml at 37C, while cultures of the parent strain produce $7x10^8$ heat-stable spores per ml at that temperature.

Growth studies of the mutants and parent strain at 26C reveal that functions such as oxidation of organic acids and formation of heat resistant spores, which are blocked at 37C, are somewhat impaired at 26C. The development of a functional tricarboxylic acid cycle and the development of refractile fore-spores is delayed for about three hours in cultures of the mutant. The mutant produces about 60% as many spores as the parent strain at 26C.

Cultures of JS22-c were shifted from 37C to 26C at various times during growth and sporulation, and the effect of these shifts on the ability of the mutant to

form heat-stable spores was determined. Cultures shifted to 26C during exponential growth or during the first hour after the end of exponential growth are able to sporulate at near normal levels, but those shifted later are impaired in ability to sporulate. Complete inhibition of sporulation occurs in cultures shifted at four or more hours after the end of growth. Similar experiments in which cultures of JS22-c were shifted from 26C to 37C at various times revealed that cultures shifted during exponential growth or during the first five hours after the end of exponential growth are greatly impaired in their ability to sporulate. Cultures shifted six hours after the end of exponential growth are able to sporulate at near maximal levels, indicating that the function of the temperature-sensitive gene product is dispensible after this time. Examination of thin sections of JS22-c cells removed from 26C cultures six hours after the end of exponential growth reveals that the cells had reached the end of stage II (septum formation) at that time.

Although cells shifted from 26C to 37C from one hour after the end of exponential growth through four hours after the end of exponential growth are unable to sporulate, the rise in pH values of these cultures indicates that they are able to form a functional tricarboxylic acid cycle. Rates of oxidative metabolism were followed in cultures of JS22-c which had been shifted

from 26C to 37C by measuring the rate of reduction of 2,6-dichlorophenolindolphenol. The rates of oxidative metabolism of cultures shifted to 37C three hours after the end of exponential growth increase after the shift, indicating that the temperature-sensitive protein is not one of the enzymes involved in oxidative metabolism. Cultures shifted at the end of exponential growth do not develop detectable levels of oxidative metabolism. These results are consistent with the hypothesis that the lesion affects a gene which has a control function that must be expressed before a functional tricarboxylic acid cycle can be formed. Since cultures shifted three hours after the end of exponential growth have high rates of oxidative metabolism but are still unable to sporulate, expression of this gene appears to be necessary for the function of at least one other indispensible event of sporulation.

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By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

ARTICLE 2

APPENDIX

INTRODUCTION

Endospores formed by bacteria of the genus Bacillus differ from vegetative cells in their morphology, their dormant physiological state, and their greater resistance to a number of adverse environmental conditions. The formation of Bacillus spores is initiated by conditions in which a nutrient essential for the growth of the organism has become limiting and then occurs as a precisely ordered sequence of biochemical and morphological events. The sporulation process is considered to be a primitive form of cellular differentiation. Since bacteria are susceptible to analysis by genetic and biochemical techniques which cannot presently be applied to differentiating cells of higher organisms, bacterial sporulation is being studied extensively as a model to promote better understanding of the process of differentiation in more complex organisms.

The nature and the number of indispensible steps involved in the formation of bacterial spores can be analyzed by studying large numbers of asporogenous mutants. Temperature-sensitive mutants are particularly

valuable for these kinds of analyses; because they make it easier for one to determine whether the mutation has affected a specific enzyme or a control gene, and they make it possible to determine the time period in which the altered gene normally functions.

The initial purpose of this project was to study the function of a sporulation-specific protease of Bacillus cereus through the use of temperature-sensitive mutants. Although the desired mutant was not obtained, five temperature-sensitive mutants were found which were blocked at an early stage of sporulation when grown at 37C. These mutants were also altered in their resistance to lysis by a previously undiscovered bacteriophage. This dissertation describes the isolation and preliminary characterization of the five mutants, the partial characterization of the newly discovered bacteriophage, and a more thorough investigation of one of the mutants.

LITERATURE REVIEW

Morphological Changes During Sporulation

The first photographic evidence demonstrating the correct sequence of the gross morphological changes that occur during sporulation was presented by Bayne-Jones and Petrilli in 1933 (8). Their observations were that one cell gives rise to one spore, that the spore arises within the cytoplasm at one end of the cell, that the spore area increases in size during the later stages of spore development, and that the terminal processes of sporulation are the development of refractility followed by release of the spore from the disintegrating cell. Recently, the development of techniques for achieving more synchronous sporulation in batch cultures and the refinement of electron microscopic techniques have made it possible to study these changes in much greater detail and to resolve the morphological development of spores into seven distinct stages (18, 20, 37, 50, 64).

During stage I the final round of DNA replication is completed, and the two nuclei condense, coalesce, and form an axial thread of chromatin which extends almost to the full length of the vegetative

cell (50). The formation of the axial filament is the first major structural change that takes place during sporulation; mutants blocked prior to this change are identical to exponentially growing cells in their appearance and are said to be blocked at stage 0 (30, 37).

During stage II a septum is formed near one pole of the cell (37). This results in the segregation of the DNA into two compartments, the mother cell and the forespore. Although the forespore is much smaller than the mother cell, both are believed to contain the same amount of DNA (64). No peptidoglycan material is deposited between the invaginating layers of membrane (37, 64). The asymetric nature of the cytoplasmic division and the absence of cross wall formation are the only major differences between forespore septum formation and binary fission (37).

After completion of the forespore septum, the forespore is engulfed or enveloped by the mother cell (stage III). When the forespore has become completely enveloped, the outer sides of the plasma membrane face inward toward each other, and the mechanism by which the cell wall material of the cortex is built or excreted is doubly provided for (64). Cells that have attained stage III continue their development to mature spores upon transfer into fresh medium and are said to be

"committed" to sporulation. Prior to this stage, cells transferred into fresh medium resume vegetative growth (20).

Cell wall material is deposited between the outer forespore membrane and the inner forespore membrane during stage IV. This cell wall material consists of two layers, an inner thin shell; the germ cell wall, and an outer thick shell; the cortex (37). During stage IV the forespore must remain associated with the mother cell in order to be capable of maturation. It is not until this stage is completed that the forespore can be separated (61).

Synthesis of the protein coat and the exosporium occur during stages IV and V. The end of stage V is characterized by a completed coat structure (37). At the end of stage V and during stage VI the spore undergoes a number of changes which have been described as ripening. The mesosomes become compact and barely visible, the refractility of the spore increases, and the spore becomes heat-resistant and impermeable to basic strains (64).

Stage VII is marked by the lysis of the sporangium and release of the mature spore into the medium (37).

Biochemical Events Associated With Sporulation

A number of enzymes and products have been associated with sporulation because they appear for the first time or in much greater quantities after the onset of sporulation (37, 51). It is not certain how many of these enzyme and products are really involved in sporulation and how many appear at this time merely because they are repressed by conditions promoting vegetative growth (37). This review will not consider all of the biochemical events which occur during sporulation but will be limited to those events for which a sporulation-specific function is known and those events which, when absent, result in a reduced frequency of sporulation.

Protease

The correlation of the time course of exoprotease synthesis in *B. licheniformis* (9), *B. cereus* (58), and *B. subtilis* (14, 62) with the onset of sporulation; the absence of one or more proteolytic enzymes from a large number of mutants blocked at stage 0 of sporulation (7, 37, 90); and high rate of protein turn-over normally observed in sporulating cells (51, 87); suggested that proteases were involved in the sporulation process.

The metal protease which is inhibited by EDTA accounts for nearly all of the extra-cellular proteolytic activity of *B. cereus* and *B. megaterium* (1) and

approximately 20% of the extra-cellular activity of B. subtilis (62). The function of this enzyme, to supply nutrients in assimilable form to the sporulating cell (7), may enhance sporulation; but the existence of mutants of B. cereus (1, 2), B. megaterium (1), and B. subtilis (33) which do not produce an active metal protease but are able to sporulate normally indicates that this enzyme is not essential to sporulation.

The serine protease, which is inhibited by phenyl methyl sulfonyl floride, appears to be the protease which is essential to sporulation. Sadoff et al. (75) have shown that the serine protease of B. cereus, in a limited proteolysis, converts vegetative aldolase to spore aldolase. More direct evidence that a functional serine protease is required for the initiation and subsequent stages of sporulation was obtained by Leighten et al. (56, 57, 76) who isolated a temperature-sensitive mutant of B. subtilis with a lesion in the structural gene coding for the serine protease. Studies of this mutant revealed that sporulation is blocked at stage 0, and the mutant is unable to produce antibiotic or sporulation-specific RNA polymerase at the restrictive temperature. The serine protease is not responsible for general protein turnover in B. subtilis (56).

Although the results Leighton et al. (56, 57) indicate that the serine protease of B. subtilis has both

intra and extracellular functions, recent studies have shown that the major intracellular serine protease of B. subtillis is different from the extracellular serine protease. Reysset and Millet (70) found that the intracellular serine protease had an absolute requirement for Ca^{++}, not found in the extracellular serine protease, and a narrower substrate specificity than the extracellular protease. Hageman and Carlton (33) found that the two serine proteases had different electrophoretic mobilities.

Although much evidence has been obtained which supports the supposition that intracellular proteolytic activity is required for sporulation, one report by Slapikoff et al. (86) appears to contradict that evidence. They reported that a strain of B. brevis which lacks detectable intra or extracellular proteolytic activity (using ^{14}C denatured protein as substrate) is able to sporulate normally without undergoing protein turnover. Aronson (1) suggested that the apparent lack of protein turnover observed may be due to the insensitivity of the method used by Slapikoff (86) for detecting small changes in turnover rates. Measurements of labled amino acid release from the metabolic pool of cells would probably be a more sensitive method to determine protein turnover (1).

Peptide Antibiotics

Several lines of evidence suggest a relationship between the production of peptide antibiotics and sporulation:

1. Specific sporulation inhibitors also inhibit antibiotic synthesis (10, 67).
2. Many pleiotrophic stage 0 mutants of B. subtilis are antibiotic negative (79).
3. A few mutants selected for their inability to produce antibiotics are asporogenous (81, 82).
4. Restoration of antibiotic production by reversion, transduction, or transformation also restores ability to sporulate (43).

The antibiotics produced by sporulation bacilli have been classified into three groups (73). Group 1 consists of edeines, basic linear peptides which inhibit DNA synthesis (52); group 2 consists of bacitracins, cyclic peptides containing amino acid condensation product which inhibit cell wall synthesis (85); and group 3 consists of gramacidins, polymixins, and tryocidins, linear (78) or cyclic peptides (72, 88) which modify membrane structure or function. The edeines (53), gramicidins (100), tyrocidins (71), and polymixins (68) are not the products of normal protein synthesis but are synthesized enzymatically. Bacitracins appear to be the products

of the proteolytic cleavage of a protein formed earlier in the growth cycle (74). Sadoff (73) has proposed that edienes function in the inhibition of DNA synthesis that occurs during stage I, that gramicidins, tyrocidins, and polymixins each have a role in the membrane alterations that occur during stage II and stage III, and that bacitracin prevents cell wall synthesis during forespore septum formation and engulfment.

It is possible that the quantities of antibiotic required in sporulation may amount to only a few molecules per cell and that the known antibiotic producing strains are mutants which overproduce products that occur in much smaller amounts in all aerobic sporulating bacilli (73). If these suggestions are correct, the high frequencies of sporulation which have been observed in mutants which produce low levels of antibiotic (82) and the apparent absence of antibiotic in some strains of Bacillus would be explained.

Alkaline Phosphatase

The alkaline phosphatase which appears during sporulation is much less susceptible to repression by phosphate than that produced by vegetative cells (47, 96). Although production of this alkaline phosphatase, like sporulation, is delayed by addition of glucose to the medium (47), and alkaline phosphatase activity is absent in some ts mutants under conditions in which

sporulation does not occur (55, 57); it is still not known whether alkaline phosphate activity is necessary for spore development or merely a by-product (47).

Tricarboxylic Acid Cycle Activity

Hanson et al. (38) observed that growing cultures of B. cereus are unable to oxidize the acetate that accumulates in the medium while sporulating cells are able to oxidize acetate at a high rate. Further study of this phenomenon revealed that several of the enzymes of the tricarboxylic acid cycle are absent from vegetative cells and that the appearance of these enzymes corresponds to the onset of sporulation (39). The appearance of high rates of acetate oxidation in cultures of B. subtilis also corresponds to the onset of sporulation although low levels of activity can be detected in vegetative cells of that strain (35). The synthesis of the tricarboxylic acid cycle enzymes, like the initiation of sporogenesis, is subject to catabolite repression (36, 91). In B. cereus, the amount of cytochromes associated with the membranes increase concomitant with the increase in activity of the tricarboxylic acid cycle (19, 54).

The results of several early studies suggested that the synthesis of a functional tricarboxylic acid cycle is an essential step in the sporulation process. Hanson et al. (38, 39) observed that the addition of

α picolinic acid to cultures of B. cereus simultaneously inhibited the synthesis of aconitase and the initiation of sporulation. When the inhibition of aconitase synthesis was reversed by addition of metal ions the medium, the inhibition of sporulation was also reversed. Szulmajster and Hanson (35, 91) isolated an asporogenic mutant which was aconitase negative and transfer of this defective gene to wild-type strains by transformation caused these strains to become asporogenic. Freese et al. have observed that a number of mutants, isolated for their inability to sporulate, have lesions in various structural genes of the tricarboxylic acid cycle (23, 24, 25).

The method of isolating asporogenic mutants and screening them for lesions in the tricarboxylic acid cycle precludes the selection of sporogenic mutants which lack a functional tricarboxylic acid cycle. To overcome this experimental limitation, Carls and Hanson (12) developed a technique for the direct isolation of mutants lacking a functional tricarboxylic acid cycle. Clones isolated by this technique were selected for their inability to oxidize organic acids rather than their inability to sporulate. However, mutants isolated by Carls and Hanson were all impaired in their ability to sporulate although three isolates were able to form spores in 1 to 10% of the viable cells. Some of these

mutants sporulated poorly while others with the same lesion sporulated well. Their observations cannot be explained at the present time.

Freese and co-workers (24) believed that the poor sporulation of tricarboxylic acid cycle mutants resulted from a deficiency of ATP during sporogenesis. However, recent studies (26, 99) have shown that the arrest of these mutants at stage I is not due entirely to an insufficient energy supply. If mutants blocked in the first half of the tricarboxylic acid cycle are supplied with a metabolizable carbon source they are able to maintain high levels of ATP but are not able to sporulate unless they are resuspended in culture fluids from post-exponential phase wild-type B. subtilis or E. coli. Mutants blocked in the second half of the tricarboxylic acid cycle are not able to sporulate even under these conditions. These observations suggest that tricarboxylic acid cycle mutants are unable to remove a suppressor(s) of sporulation from the growth medium.

Cortex and Coat Synthesis

Two of the events that occur only during sporulation for which a sporulation-specific function is known are the synthesis of the cortical peptidoglycan and the synthesis of the spore coat protein. Although the cortex is made up of peptidoglycan (97, 98) there is evidence that it differs in chemical structure from

the vegetative cell wall and the germ cell wall. The cortex is hydrolysed during germination, while the germ cell wall persists and becomes the cell wall of the germinated spore (64). The cortex, which is sensitive to lysozyme, differs from the germ cell wall and vegetative cell wall which are not (97, 98). The cortex is synthesized immediately after the closure of the forespore septum. It is synthesized de novo, not from cell wall turnover (94). The number and nature of unique enzymes involved in the synthesis of the cortex are not known, due to the difficulties in recognizing and enriching for mutants devoid of these enzymes (37).

The spore coat of B. cereus T contains primarily protein (6) and consists of two morphological layers (5). Recent studies have shown that the proteins in both coat layers are identical and that there may be only a single major species of polypeptide in the spore coat (5). There appear to be two major biochemical events associated with coat formation in B. cereus T (6). The first is the initiation of coat polypeptide precursor synthesis shortly after the termination of exponential growth. The second is the incorporation of cystine into the spore coat at the time of the formation of well-defined coat layers. The cystine appears to be involved in the formation of intermolecular disulphide bonds (5). Studies of exoprotease regulatory mutants which over or

underproduce coat protein suggest that coat synthesis is subject to regulation by catabolic repressors which are separate from but metabolically related to the catabolic repressors for exoprotease synthesis (4).

Dipicolinic Acid Synthesis

The synthesis of dipicolinic acid (DPA) like the synthesis of the spore cortex and the spore coat is considered to be a sporulation-specific event. DPA is absent from vegetative cells, but it is present in high concentration in all bacterial endospores, presumably as a calcium chelate (64, 69). Calcium and DPA are considered to play important roles in the heat-resistance of spores. Mutants which contain low levels of DPA or no DPA produce spores with reduced heat-resistance (3, 21, 28, 34). Starvation of cells for calcium also results in the production of heat-sensitive spores (21). DPA synthesis occurs during cortex formation and maturation of the sporangia (stages IV to VI) (40, 93) and is accompanied by incorporation of calcium into the endospores (11, 40). Analysis of mutants has shown that some of the enzymes of the lysine biosynthetic pathway participate in DPA synthesis (21, 28). The first enzyme of that pathway, aspartakinase, loses its sensitivity to feedback inhibition by lysine about two hours before DPA synthesis begins. This change appears to be due to an alteration of the pre-existing vegetative enzyme (21),

but other enzymes in the pathway do not appear to be altered (13, 21). DPA synthase, which catalyzes the conversion of dihydrodipicolinic acid to DPA, can only be detected in sporulating cultures just prior to the onset of DPA accumulation (13).

Regulation of Sporulation

Catabolite Repression

Massive sporulation is suppressed as long as the growth medium contains an excess of rapidly metabolizable carbon and nitrogen sources (79). Depletion of these energy and nitrogen sources appears to be a factor involved in the initiation of sporulation (64). With B. subtilis, the probability that sporulation will occur depends on the nature and concentration of the carbon source and on the nature of the available nitrogen source. With B. megaterium sporulation occurs whether either the carbon or the nitrogen source is removed or depleted (81). These observations strongly suggest that sporulation is under the control of catobolite repression (81). Freese and co-workers (24) have identified at least three compounds, not interconvertible in certain mutants, which are involved in the repression of sporulation. They are D-glucose-6-phosphate, L-α-glycerol-phosphate, and some compound(s) derived from L-malate.

The mechanism of catabolite repression in Bacillus species is not understood. Cyclic 3', 5'-monophosphate

(cAMP) is the chemical messenger that signals low energy levels in E. coli and it is required for the transcription of catabolite-repressible genes in that strain (66). Attempts to detect cAMP in Bacillus species have not been successful (48, 84), and therefore it is likely that an alternative chemical is used in Bacillus. Although the nature of the signal molecule in Bacillus has not been determined, its presence or absence is dependent on the energy charge of the cells (46).

Changes in Nucleic Acid Synthesis

Replication of DNA is not irreversibly arrested during stage I or stage II in either B. subtillis or B. cereus. Transfer of these strains into fresh medium during stage I or stage II results in renewed vegetative growth. However, cells that have attained stage III have become committed to sporulate and cannot initiate DNA synthesis (20, 37). Although the reason for this irreversible cessation of DNA replication is not known, it is probably not due to the state of the DNA in the axial chromatin filament but is more likely due to the biochemical changes associated with the membrane rearrangement that take place during engulfment (37).

A number of studies have shown that gene transcription is altered at the onset of sporulation. Hussey et al. (44) observed that the incorporation of radioactive

uracil into B. subtilis 30 s and 50 s ribosomal subunits stops during early sporulation, indicating that ribosomal RNA is not synthesized during sporulation. Doi and Igarashi (16) used DNA-RNA hybrid competition studies to demonstrate that genes are transcribed during sporulation which are not expressed during growth or germination. They observed that a 15-30% fraction of labled sporulation mRNA from B. subtilis was able to hybridize with DNA in the presence of an 150 fold excess of unabled exponential phase or germination phase mRNA. Using the same technique, Di Cioccio and Strauss (15) found that unique mRNA species occur at specific times during the sporulation process, that significant transcription occurs on the light DNA strand, and that only about 20% of the vegetative RNA species are turned off during sporulation. Sumida-Yasumoto and Doi (89) have established that differential transcription occurs during sporulation in a sequential manner and that spore-specific genes are present in both complementary strands of DNA. Early in sporulation (stage 0 to I) only the light strand of DNA is specifically transcribed for sporulation genes but in stages III and IV both strands are transcribed.

The first evidence that the changes in transcription that occur during sporulation are due, at least in part, to a change in the RNA polymerase was presented by Losick and Sonenshein (60). They compared

the ability of RNA polymerase obtained from vegetative cells of B. subtilis with that of RNA polymerase obtained from sporulating B. subtilis to transcribe the DNA of øe, a phage which only replicates in exponetially growing cells. The results of this study showed that the RNA polymerase from exponentially growing cells actively transcribes øe DNA while that from sporulating cells is inactive with the same DNA. Leighton et al. (56, 57) have proposed that the change in the template specificity of B. subtilis RNA polymerase was due to a specific cleavage of the β subunit of the polymerase by a serine protease. Their evidence was derived from observations on their temperature-sensitive serine protease mutant which failed to modify the polymerase at the restrictive temperature but was able to modify the polymerase at the permissive temperature. Their strongest evidence was the observation that the template specificity of RNA polymerase was altered when the mutant was shifted to the permissive temperature in the presence of inhibitors of protein synthesis (56). Millet et al. (63) have observed a similar modification of the β subunit upon treatment of the B. subtilis RNA polymerase with intracellular serine protease from B. megaterium.

Recent evidence obtained by Linn et al. (59) indicates that the modification of the β subunit by the serine protease is not responsible for the observed changes

in template specificity but is an artifact of the procedures used by Leighton et al. (56, 57) and Millet et al. (63) to obtain and purify the polymerase. Linn et al. (59) observed that no modification takes place when the cells are harvested rapidly at OC, immediately washed with a salts medium containing the serine protease inhibitor phenyl methyl sulfonyl floride (PMSF), and if the entire RNA polymerase purification procedure is performed in the presence of PMSF. Although no proteolytic modification occurs by this procedure the change in template specificity is still evident. This change is due, partially, to a loss in activity of the δ subunit which occurs during the first two hours of sporulation (59). The core enzyme which contains no δ activity not only loses the ability to transcribe øe mRNA, but it also loses the ability to transcribe rRNA in vitro (45). Greenleaf et al. (30, 31) have purified a polypeptide with a molecular weight of 70,000 daltons which binds to the core polymerase. This polypeptide, which first appears during the third hour of sporulation, appears to be sporulation-specific. Thus, the most recent evidence suggests that the change in template specificity of the RNA polymerase during sporulation is due to loss of δ activity and the acquisition of a new polypeptide subunit during sporulation.

Translational and Post-Translational Control

Fortnagel and Bergman (22) have demonstrated by gel electrophoresis that there are significant quantitative differences between the ribsomal proteins of vegetative cells and those of sporulating cells and that at least one new protein is present in ribosomes of sporulating cells. They also observed that vegetative cells and ribosomes from vegetative cells are sensitive to the antibiotic fusidic acid, while sporulating cells and ribosomes from sporulating cells are resistant. Graham and Bott (Bacteriol. Proc. P. 39, 1974) studied mutants resistant to antibiotics that act at different ribosomal sites and found that some of the mutants grow normally in the presence or absence of the antibiotic but are not able to sporulate; others grow normally but are only able to sporulate in the absence of the antibiotic. The differential effects of these antibiotics on growth and sporulation strongly suggests that there are changes in the translational mechanisms of the ribosomes during sporulation. The appearance of some unique species of tRNA in spores (95) suggests that these may also be involved in translational control during sporulation.

The best evidence for post-translational control is the demonstration by Sadoff et al. (75) that the serine protease converts vegetative aldolase of B. cereus to the spore form in vitro, and the demonstration by Sadoff

and Celikkol (74) that antibiotic is formed by the cleavage of a pre-existing cellular protein by the serine protease.

Temperature-Sensitive Asporogenous Mutants

A valuable tool for studying gene function during sporulation, which has only recently been utilized, is the temperature-sensitive (ts) asporogenous mutant. Temperature-sensitive mutants can be used to obtain information which totally negative mutants are unable to provide. Point mutants which are ts enable one to determine whether the mutation resides in a structural gene or a control gene; they also permit one to control the phenotypic expression of a single gene product by shifting the temperature of the cultures. From these temperature-shift experiments, the time period during sporulation in which the function of the mutated gene is indispensible can be determined (17, 42).

Szulmajster and co-workers (32, 65, 90) have isolated a ts mutant of B. subtilis (ts-4) which grows normally at both permissive (30C) and restrictive (42C) temperature but is blocked at stage 0 at 42C. The mutant does not produce antibiotic, has low rates of RNA and protein turnover, and is defective in the late enzymes involved in DPA synthesis. Experiments in which the capacity of the mutant to sporulate in cultures shifted

from 42C to 30C or from 30C to 42C at various times during growth and sporulation revealed that the temperature-sensitive event occurs in an early stage of spore development and that the loss in sporulation capacity at 42C is irreversible if the shift is made more than one hour after the onset of sporulation. Although the nature of the genetic lesion in the ts-4 mutant is not known, it has been found that stationary phase cells have a low capacity for protein synthesis at 42C, due to the presence of large amounts of an mondialyzable inhibitor which affects amino acylation of phenylalanyl tRNA. The inhibitor is not specific to stationary ts-4 cells. It is also found in exponentially growing cells of the wild-type. The critical factor appears to be in the large quantities of inhibitor found in the mutant during stationary phase.

Hitchins and Sadoff (41, 42) have isolated a ts mutant (TH-14) of Bacillus megaterium which has a lesion in a gene essential to the formation of both cell division septa and forespore septa. DNA synthesis continues when the cells are shifted to the restrictive temperature, and the mutant forms multinucleate aseptate filaments. The sporulation frequency of TH-14 at the restrictive temperature is approximately 10^{-5}. Experiments in which cultures were shifted from permissive to restrictive temperature during sporulation have shown

that the septation and engulfment stages are particularly sensitive to the temperature shift. Although there is no qualitative change in extractable membrane proteins when the mutant is grown at the restrictive temperature, there appears to be a partial derepression of a membrane protein(s) with a molecular weight of approximately 80,000, and there is a reduced content of a small molecular weight protein(s). Numerous spherical inclusions, recently identified as glycogen, are present in the cytoplasm of TH-14 at the restrictive temperature. These are concentrated at only one pole of each filament.

Leighton and co-workers have isolated ts mutants of B. subtilis which are blocked in specific functions believed to be involved in the sporulation process (55, 56, 57, 76, 77). One of these mutants (ts-5) has a genetic lesion in the structural gene for a serine protease. When grown at the restrictive temperature, this mutant is blocked at stage 0. Results of temperature-shift experiments with ts-5 indicated that the serine protease activity is required throughout the sporulation sequence. The evidence from these studies indicated that the serine protease is not responsible for the general intracellular protein turnover.

Other mutants isolated by Leighton include ts rifampin-resistant which are also ts RNA polymerase

mutants which appear to block sporulation at uniquely different time periods including stages 0, II, and IV. Some of these mutants have very long temperature-sensitive periods while others have very short and discrete temperature-sensitive periods (55). One of these mutants (ts-14) has been studied in detail. The mutation in ts-14 is expressed during the middle third of the sporulation sequence. Early events such as protease and antibiotic synthesis are not affected by growth at the restrictive temperature while alkaline phosphatase synthesis is blocked (55). Morphological studies of ts-14 cells grown at the restrictive temperature have revealed that abnormal synthesis of cell wall occurs along the forespore membrane; this prevents the engulfment process and causes abortive sporulation to occur (77).

Bacteriophage and Sporulation

The expression of the lytic cycles of several bacteriophage of *Bacillus* species appears to be related in some way to the sporulation properties of the host strains. While studies of this particular phenomenon are not extensive, in at least two instances phage production has been associated with an impairment in the ability of the host strain to sporulate. Thorne (92) observed that lysogenic cultures of *Bacillus subtilis* W-23-S^r produce very low titers of phage SP-10 when they are grown in a medium that produces high yields of

spores, and they produce high titers of phage when they are grown in a medium that produce low yields of spores. Ito and Spizizen (49) found that bacteriophage Ø2 is not able to replicate in mutants of that strain blocked in early sporulation functions. They have suggested that an alteration in the ribonuceic acid polymerase in the mutants or an alteration in the membranes involved in phage restrictions may be responsible for this phenomenon.

Goldberg and Gollakota (29) have isolated a bacteriophage from B. cereus T which may be related to sporulation in an entirely different way. This phage does not produce mass lysis of the bacterial culture until after the onset of sporulation, and the production of this phage is inhibited by agents that inhibit sporulation. These observations suggest that replication of this phage may be dependent on changes that occur in the cells during sporulation.

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ARTICLE 1

PRODUCTION OF BACTERIOPHAGE BY TEMPERATURE-SENSITIVE SPORULATION MUTANTS OF *BACILLUS CEREUS* T

By

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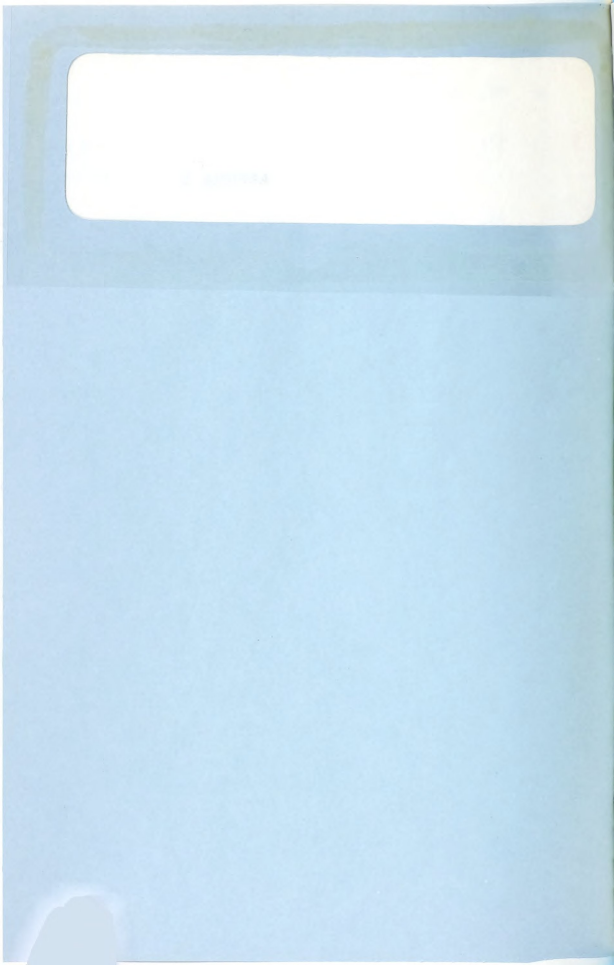

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Production of Bacteriophage by Temperature-Sensitive Sporulation Mutants of *Bacillus cereus* T[1]

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Five temperature-sensitive sporulation mutants of *Bacillus cereus* T have been isolated. These mutants are blocked at stage 0 of sporulation at the restrictive temperature (37 C) but are able to sporulate at nearly normal frequencies at the permissive temperature (26 C). A bacteriophage that forms a stable lysogen in the parent strain is induced at increased frequencies in the mutants. This induction is accompanied, in some of the mutants, by a reduction in immunity to the phage. Revertants, selected for their ability to sporulate normally at both temperatures, lose their ability to produce high titers of the phage. In addition to this lytic phage, an apparently defective phage has been found in lysates of the mutants. Strains cured of the plaque-forming phage still carry the defective phage. Comparisons of physical and biological properties of the plaque-forming phage with those of the two *Bacillus cereus* phages most similar to it have shown that this phage is not identical to either of them. The maximal titer of phage produced in cultures of the parent strain is about 10^3 plaque-forming units (PFU) per ml at both temperatures. The maximal titers of phage produced by the mutant are 4×10^9 PFU/ml at 37 C and 7×10^8 PFU/ml at 26 C. Both mutant and parent strains release over 90% of the phage they produce after the onset of stationary phase.

Preliminary investigations of five temperature-sensitive (ts) sporulation mutants of *Bacillus cereus* T indicated that a sporadically induced lytic agent was present in the culture supernatant fluids of all five mutants. Through its ability to form discrete plaques on lawns of sensitive bacteria and through observation of the particles under the electron microscope, we were able to identify the lytic agent as a bacteriophage.

The present paper describes the isolation and preliminary characterization of the ts mutants, some physical and biological properties of the phage which distinguish it from similar phages previously studied (1, 6, 16, 17; Goldberg and Gollakata, Bacteriol. Proc. p. 47, 1960; Gollakata and Halvorson, Bacteriol. Proc. p. 86, 1960), and some aspects of the phage-host cell relationship in the mutants and in the parent strain.

MATERIALS AND METHODS

Bacterial strains and cultivation. The strain from which our mutants were derived was a tryptophan auxotroph of *B. cereus* T, isolated in our laboratory. The tryptophan requirement was a convenient marker for verifying the origin of the sporulation mutants. *B. cereus* NRRL569 and *B. cereus* ATCC6464 UM4, which are sensitive to bacteriophage CP-53, and *B. cereus* ATCC6464, which carries phage CP-53, were obtained from Curtis B. Thorne (University of Massachusetts, Amherst). Stocks of all bacterial strains were stored in 60% glycerol buffered with 0.1 M sodium phosphate (pH 7.0) at −20 C. These stocks were transferred at 6-month intervals. The stock spore suspensions, from which inoculations were made routinely, were grown either in G medium (8) or CH medium at 26 C, washed three times with distilled water, and stored in distilled water at 4 C.

Chemicals. The *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) was purchased from the Aldrich Chemical Co., Milwaukee, Wis. The acridine orange was purchased from Allied Chemicals, New York. The 2,3,5-triphenyltetrazolium chloride was obtained from General Biochemicals Inc., Chagrin Falls, Ohio. Pancreatic ribonuclease (RNase) (grade A) was a product of Sigma, St. Louis, Mo. Pancreatic deoxyribonuclease (DNase) was obtained from the California Corporation for Biochemical Research, Los Angeles, Calif.

Media. Skim milk agar was used for detection of asporogenic mutants, which form translucent colonies on this medium, whereas sporulating *B. cereus* form

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white colonies. The exoprotease activity of the mutants was simulataneously scored on this medium, which consisted of 0.3% skim milk (Carnation), 0.02% yeast extract (BBL), and 1.5% agar (Difco). The skim milk was sterilized separately and added just before the plates were poured. Modified G medium was prepared by the method of Hashimoto et al. (8). CH medium was the same composition as modified G medium except 0.6% vitamin-free casein hydrolysate (NBC) was substituted for 0.2% yeast extract. Bromocresol purple medium had the same salt and yeast extract concentrations as modified G medium but contained only 0.2% glucose instead of the usual 0.4%, and 0.015 g of bromocresol purple (Difco) per liter. NBY medium consisted of 0.8% nutrient broth (Difco) and 0.3% yeast extract (15). The soft agar used in overlays contained 0.5% agar. All media except the NBY medium were supplemented with 20 μg of L-tryptophan per ml.

Isolation of mutants. An effort was made to isolate temperature-sensitive mutants which were protease negative and asporogenic at the restrictive temperatures. Nutrient broth cultures, started from spores heat-shocked at 70 C for 30 min were harvested in mid-exponential growth phase by centrifugation at 12,000 $\times$ *g* for 10 min at 4 C. The cells were washed twice with an equal volume of 0.05 M Tris[hydroxymethyl]aminomethane-maleic acid buffer, pH 6.0, suspended in the same buffer containing 200 μg of NTG/ml, and shaken for 30 min at 30 C. Normally 80 to 90% of the cells were killed by this procedure. The surviving cells were washed with sterile G medium, resuspended in G medium, and grown at 37 C on a New Brunswick model G76 water bath-shaker at 240 rpm. The culture was observed periodically under the phase microscope until most cells reached the forespore stage and became irreversibly committed to sporulate (5). At this time, glucose and yeast extract were added to final concentrations of 0.4 and 0.2%, respectively. This supplement permitted the growth of asporogenous mutants while most of the cells in the culture were completing sporulation. When free spores were observed, the culture was harvested, washed, and resuspended in sterile G medium. A 5-ml amount of these cells was used to inoculate 50 ml of fresh G medium, and the enrichment for asporogenic mutants was repeated. On completion of the second enrichment, the cells and spores were harvested by centrifugation, washed with sterile 0.1 M phosphate buffer (pH 7.0), and suspended in 60% glycerol. Samples of cells in glycerol were stored at −20 C.

Dilutions of these samples which produced 50 to 100 colonies per plate were spread on skim milk agar, and the plates were incubated at 37 C. After 12 h of growth at 37 C the colonies were examined for protease activity, and those having no apparent activity were restreaked on fresh skim milk medium. The plates were then placed in the 37 C incubator for another 12 h, and after 24 h total incubation replicate plates were made. These were exposed to chloroform for 5 min and incubated at 37 C for 12 h. Colonies which produced spores after 24 h were not sensitive to the chloroform treatment and were able to grow on the replicate plates, whereas asporogenous mutants failed to grow on the replicate plates. All mutants which were asporogenic at 37 C were restreaked and grown at 26 C to test their ability to sporulate at lower temperatures.

Isolation of revertants. Cultures of the ts mutants were grown in modified G medium at 37 C. A sample of early stationary-phase cells was diluted and spread on nutrient agar plates for determination of the total viable count. Forty-eight hours after inoculation, a second sample was removed and placed in a screw-cap vial which was submerged in a 70 C water bath for 30 min. The surviving spores were spread on skim milk medium and incubated at 37 C. Revertant colonies could be distinguished after 24 h of growth by their white appearance. The reversion frequency was determined by dividing the number of revertants per milliliter by the total viable count per milliliter.

Electron microscopy. Phage were produced on plates in soft agar overlays. They were prepared for electron microscopy by scraping the soft agar layer from the plates, mixing in a small volume of 0.1 M sodium phosphate buffer (pH 7.0), and sedimenting at 12,000 $\times$ *g* to separate the cells and the agar from the phage. Alternatively, the supernatant fluids of broth cultures were spun at 125,000 $\times$ *g* for 2 h, and the resultant phage pellet was suspended in phosphate buffer. The phage were stained with 0.5% phosphotungstic acid (PTA) and were observed and photographed under a Philips EM-300 electron microscope.

Propagation and assay of phage. Broth cultures of the ts *B. cereus* mutant JS22 lyse spontaneously due to the induction of a lysogenic bacteriophage (LP-22). The initial propagation of LP-22 was as follows. Mutant JS22 was grown from a spore inoculum in 100 ml of CH medium at 37 C for 10 h into the stationary phase. The cells were pelleted by centrifugation at 12,000 $\times$ *g* for 10 min in a Sorvall RC2-B centrifuge. The supernatant fluids were then subjected to 125,000 $\times$ *g* in an International preparative ultracentrifuge (model B60) for 2 h at 4 C to pellet the phage particles. The phage were washed with 1% peptone (Difco), resuspended in 10 ml of 1% peptone, and stored at 4 C over chloroform.

Phage CP-53 was induced in *B. cereus* ATCC6464 by the method of Yelton and Thorne (16) and was stored in 1% peptone at 4 C.

Spontaneously cured strains of mutant JS22 were isolated in the following manner. Flasks (500 ml) containing 50 ml of nutrient broth were inoculated with 3×10^6 heat-shocked spores per ml and incubated at 37 C. After approximately four generations, samples were removed, diluted, and spread on nutrient agar plates. The plates were incubated at 37 C for 12 h, after which time smooth colonies were selected and tested for sensitivity to the phage and for phage activity in their culture supernatants.

Phage isolates were assayed by standard techniques using soft nutrient agar overlays seeded with cells from a cured strain of JS22 (JS22-C). Phage CP-53 was assayed by the method of Yelton and Thorne (16) using lawns of *B. cereus* ATCC6464 UM4.

Preparation of antisera. Antisera to the newly isolated phage, LP-22, and to CP-53 were prepared in

the same manner. A 2-ml amount of phage suspended in sterile saline at a concentration of 10^{10} plaque-forming units (PFU)/ml was mixed with an equal volume of Freund complete adjuvant and injected subcutaneously into rabbits. Booster injections of 10^{10} PFU/ml in Freund incomplete adjuvant were given at 3- to 4-day intervals. A total of six injections was given. The rabbits were bled from the ear vein 1 week after the last injection.

The antisera and the preimmune sera taken from each rabbit before the first injections were sterilized by filtration through membrane filters (pore size 0.45 μm; Millipore Corp.), heated at 56 C for 30 min to destroy the complement, and stored at −20 C.

Nucleic acid staining. Suspensions of phage LP-22 (10^{11} PFU/ml) in 0.1 M sodium phosphate buffer (pH 7.0) were treated with 0.1% pancreatic RNase and 0.2% pancreatic DNase plus 10^{-3} M Mg^{2+} at 37 C for 2 h. The phage were then pelleted by centrifugation at 125,000 × *g*, washed with 0.1 M sodium phosphate buffer, and resuspended in buffer at a concentration of 10^{11} PFU/ml. Four drops of the phage suspension were added to each of several slides. The slides were treated with Carnoy fixative (2), then with RNase, DNase, or buffers containing no nucleases, and stained with acridine orange as described by Bradley (2). The stained preparations were observed under a Blak-Ray long-wave ultraviolet lamp (Ultra-Violet Products Inc., San Gabriel, Calif.).

Ultraviolet irradiation. The phage were suspended in 10 ml of 0.1 M sodium phosphate buffer (pH 7.0) in a glass petri dish 9 cm in diameter and irradiated with a 30-W General Electric germicidal ultraviolet lamp at a distance of 40 cm. During exposure, the phage suspension was stirred constantly on a magnetic stirrer.

Time course of phage production. The time course of phage production and maximal PFU per milliliter were measured in cultures of both JS22 and the parent strain at 26 and at 37 C. Flasks (500 ml) containing 100 ml of CH medium were inoculated with 3×10^6 spores per ml (heat-shocked at 70 C for 30 min) and incubated on the water bath-shaker at 240 rpm. Growth was followed by measuring absorbance at 620 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. At various intervals, samples were removed and subjected to centrifugation at 12,000 × *g* for 15 min to remove the cells. The supernatant fluids were treated with a few drops of chloroform to kill any residual cells, diluted, and titrated by the agar overlay technique with strain JS22-C as the indicator strain.

RESULTS

Isolation of mutants. The original screening was for temperature-sensitive protease mutants with impaired ability to sporulate at 37 C, the restrictive temperature. However, of 25,000 colonies screened for protease activity, only 20 protease mutants were observed. None of these mutants was completely blocked in exoprotease secretion, and all but one sporulated normally. In addition to the protease mutants, 924 mutants were obtained which had translucent colonies on skim milk agar, rather than the normal white colonies.

Since early-stage asporogenic mutants of *B. subtilis* have been observed to produce translucent colonies (13), we investigated the ability of our translucent mutants to form spores. Agar plates (24 h) having both translucent and white colonies were replicated, and the replicated plates were exposed to chloroform for 5 min and incubated at 37 C for 4 h. In every replication under these conditions, the translucent colonies were unable to grow; their sensitivity to chloroform indicated their inability to sporulate.

Five of the asporogenous mutants (JS22, JS23, JS25, JS26, JS29) were temperature sensitive. These produced translucent colonies with no spores at 37 C and white sproulating colonies at 26 C. All of the asporogenous mutants, including the ts mutants, appeared to be normal in protease activity, as indicated by measurement of the zones of proteolysis around the colonies on skim milk agar.

The five ts mutants and ten of the other asporogenous mutants were examined under the phase microscope. The cells of all of these mutants were identical in size and shape to vegetative cells of wild-type *B. cereus* T, and all of them appeared to be blocked in the sequence of events leading to sporulation at some point prior to septum formation.

Properties of the temperature-sensitive mutants. Sporulation of the parent strain and of the ts mutants was measured in a medium containing casein hydrolysate to enhance spore yields of the mutants at the permissive temperature. The viable count (V) of cells grown at 26 or 37 C was determined by diluting the cells in saline and plating them on nutrient agar. The spore counts (S) were determined by heating the cell suspensions at 70 C for 30 min prior to dilution and plating. The S/V ratios of the parent strain were approximately 1.0 in cultures grown at both 37 and 26 C. The S/V ratios of the mutants were approximately 10^{-5} in cultures grown at 37 C and 0.6 to 0.75 in cultures grown at 26 C. These experiments were repeated with two of the ts mutants at 23 and at 20 C to determine whether 100% sporulation could be achieved. No significant increase in sporulation frequency was observed with either mutant at the lower temperatures.

Thin sections of cells of mutant JS22 grown at 37 C for 30 h were examined by electron microscopy. All cells observed appeared to be blocked at stage 0 (5). No evidence for the formation of an axial filament or a forespore septum was seen in any cell.

Preliminary growth studies of strain JS22 at 37 C indicated that the maximal pH of the medium in late stationary phase was consistently lower than the maximal pH of parallel cultures of the parent strain; this suggested the possibility that the tricarboxylic acid cycle was nonfunctional in the mutants at the restrictive temperature. The ts mutants were screened for their abilities to produce a functional tricarboxylic acid cycle at 26 and 37 C by a modification of the technique of Carls and Hanson (4). Each mutant was streaked on two plates of solid G medium containing 0.015 g of bromocresol purple per liter. One plate was incubated at 26 C and the other at 37 C. The plates incubated at 26 C changed color from purple to yellow within 10 h of incubation, due to the accumulation of acids. After 48 h of incubation the pH of the medium had increased to 6.8, and the plates were again purple in appearance. Identical results were obtained with the parent strain. The plates incubated at 37 C turned yellow after 8 h, but only the control plates (streaked with the parent strain) became purple after 48 h. The plates streaked with the mutants remained yellow even after 72 h. These observations suggest that the tricarboxylic acid cycle is functional in the mutants at 26 C but not at 37 C.

The reversion frequency of strain JS22 to sporogenesis at 37 C was approximately 10^{-7}. All revertants obtained carried the *trp*$^-$ marker which was resident in the mutants.

Discovery of phagelike particles in strain JS22. To find the growth medium which promoted optimal sporulation of the mutants at 26 C, JS22 was grown in a series of media in which varying concentrations of casein hydrolysate were substituted for the 0.2% yeast extract in G medium. When the data from three separate experiments were analyzed, a significant variation in numbers of viable cells per milliliter, numbers of heat-resistant spores per milliliter, and in S/V ratios was observed (Table 1). Preliminary growth studies of the same mutant at 37 C revealed that the growth patterns of the mutant were also highly variable. In each experiment the growth medium temperature, aeration, and inoculum were identical. This variability was not apparent in parallel studies with the parent strain.

The observed variability might have been due to a sporadic event such as spontaneous initiation of the lytic cycle of a lysogenic or carrier phage, providing that an immunity change had occurred in these cells. We reasoned that, if this were true, supernatant fluids of stationary-phase cultures of strain JS22 might produce detectable lysis on lawns of JS22 cells.

TABLE 1. *The extent of sporulation at 26 C of JS22 in media containing G salts, 0.4% glucose, and varying concentrations of casein hydrolysate*

Medium	V[a]	S[a]	S/V
0.2% CH	1.13×10^8	1.15×10^7	0.102
	7.50×10^7	3.00×10^6	0.040
	1.27×10^8	1.36×10^7	0.107
0.4% CH	5.47×10^7	6.57×10^6	0.120
	1.23×10^8	7.00×10^6	0.057
	1.68×10^8	2.05×10^7	0.122
0.6% CH	1.67×10^8	1.03×10^8	0.612
	2.76×10^8	2.13×10^8	0.772
	6.33×10^7	4.10×10^7	0.648
0.8% CH	2.65×10^8	1.26×10^8	0.476
	4.57×10^7	1.10×10^7	0.241

[a] V = total viable cells per milliliter after 48 h.
[b] S = heat-resistant spores per milliliter after 48 h.

Plates overlaid with soft agar seeded with JS22 cells were spotted with supernatant fluid from broth cultures of JS22 and incubated at 37 C. After 15 h, lawns on the control plates, to which no supernatant fluid was added, and on the plates spotted with supernatant fluid all had a mottled appearance. In this same time period, lawns on plates seeded with the parent strain were homogeneous in appearance. The soft agar layers were scraped from the plates with mottled lawns and mixed with a small volume of sodium phosphate buffer. The mixtures were spun at low speed in a centrifuge to pellet the agar and the cells. The supernatant fluid was concentrated in the ultracentrifuge, stained with PTA, and examined under a Philips EM-300 electron microscope. Phage-like particles of varying morphologies were observed (Fig. 1).

Phage morphology. The first and predominant morphological type, (Fig. 1A) has a head 65 to 70 nm in diameter and a long tail 200 nm in length and 10.5 to 11.5 nm in diameter; the base plate at the end of the tail is triangular and usually appears to have a thin strand projecting from it. This particle is very similar to phage CP-53 (17) in appearance. In the course of examination of PTA-stained preparations, other phage particles with heads 65 to 70 nm in diameter and unusual tail structures were observed (Fig. 1B-D). The tails were 8 to 9.5 nm in diameter and variable in length. A variable number of plates were found at irregular inter-

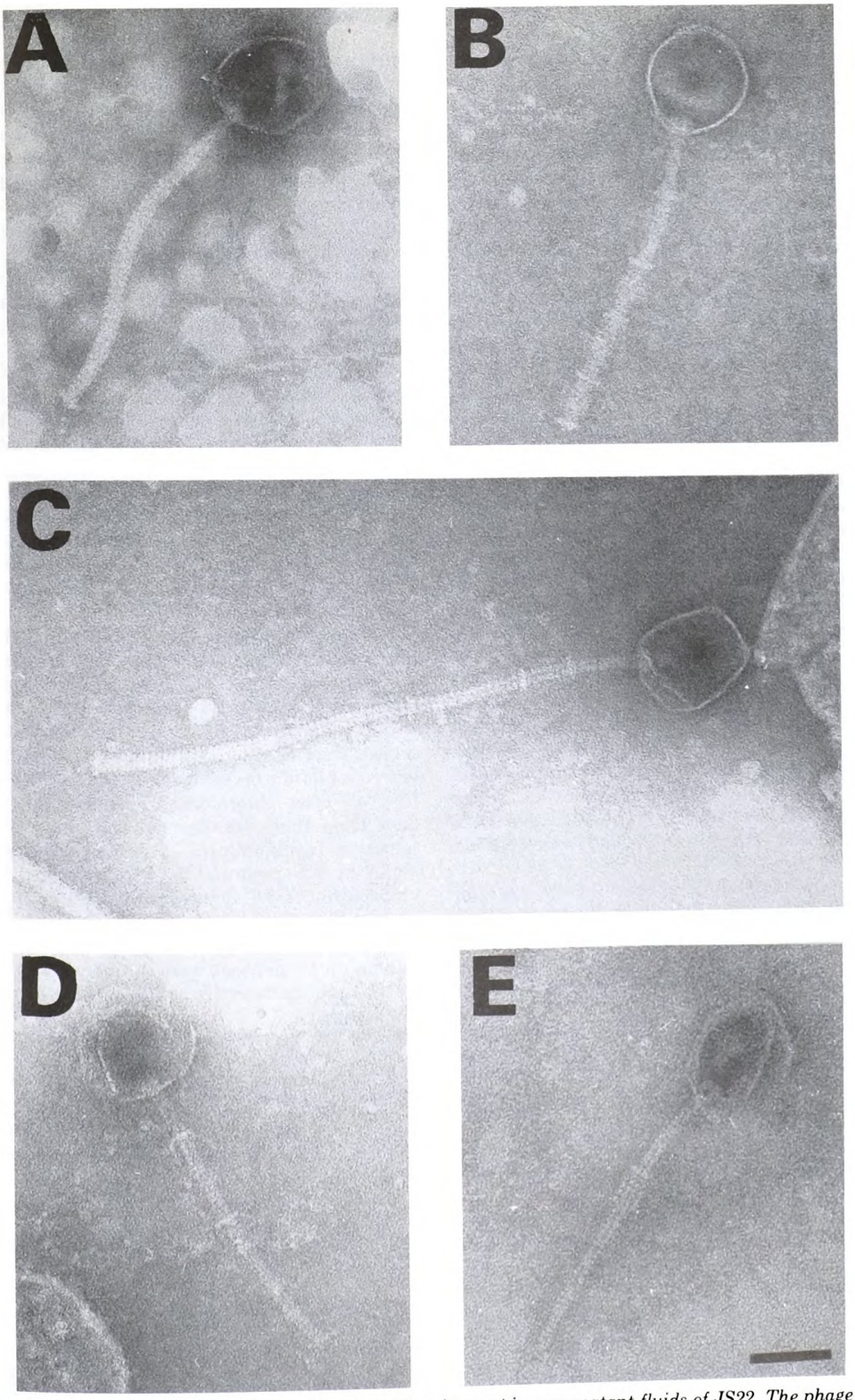

FIG. 1. *Electron micrographs of the phage particles observed in supernatant fluids of JS22. The phage were stained with PTA. The magnification is the same in all photographs, and the bar in the bottom right corner represents 50 nm.*

vals along the tails and perpendicular to the tails. Some of these plates had fine strands extending from them. End plates, when present, were identical to those of the first type. A third variety of phage particle with measurements the same as the second type was observed only in cultures cured of lytic phage (Fig. 1E). The phage shown in Fig. 1E are similar in structure to previously described defective phages of *Bacillus* species (2).

Sensitivity of the ts mutants to phage. Tests were made of the sensitivities of all of the ts mutants, the parent strain, two revertants (designated JS22-R1 and JS22-R2, respectively), and a cured derivative of strain JS22 to supernatant material from JS22 cultures. JS22 was apparently insensitive to the phage contained in the supernatant fluids, but all of the other ts mutants and the cured stocks of strain JS22 were lysed by the phage. The JS22 plates all had the same mottled appearance whether phage were added or not. The parent strain and revertants appeared to be slightly sensitive to the phage. The control plates of the other four mutants all exhibited spontaneous lysis, but rather than appearing mottled like the lawns of strain JS22 these lawns contained discrete plaques. Strain JS29 produced clear plaques, whereas the others produced turbid plaques. The experiment was repeated with supernatant fluids from each of the other mutants and from the cured derivative of strain JS22. The results emphasize the difference between JS22 and the other four mutants which were isolated (Table 2).

Development of a plaque assay system. Colonies of strain JS22 that were smooth in appearance in comparison with the normal JS22 colonies were picked from nutrient agar plates and screened for the presence of phage in their culture supernatant fluids and for sensitivity to the phage. Two strains (JS22-C1 and JS22-C2) were found that were devoid of phage activity in culture supernatant fluids and were sensitive to phage from strain JS22. Both strains carry the *trp*$^-$ marker and are identical to strain JS22 in terms of sporulation.

The plaques formed in lawns of these cured strains varied in size from pinpoint to 0.75 mm in diameter. They were irregular in shape and highly turbid in appearance. The plaques were more readily seen if lawns were stained with 0.1% 2, 3, 5-triphenyltetrazolium (12).

The efficiency of plaque formation with temperature was determined by adding equal amounts of phage to overlays seeded with strain JS22-C2 and incubating the plates at several temperatures. The efficiency increased with temperature in the range of 26 to 37 C and decreased by about 50% when the temperature was increased to 42 C. The optimal temperature for plaque formation appeared to be near 37 C. Plaque morphology did not vary with temperature of incubation.

Phage stability. The stability of phage LP-22 in chloroform was tested. A 10-ml sample of culture supernatant fluid from strain JS22 was titrated, treated with 1.0 ml of chloroform with constant agitation for 1 min, and titrated a second time. The titers were 1.35×10^8 PFU/ml before chloroform treatment and 1.40×10^8 PFU/ml after chloroform treatment. It is evident from these results that the phage is not sensitive to chloroform. Stock phage suspensions in 1% peptone have been stored over chloroform at 4 C for as long as 2 months with no loss in activity.

Screw-cap vials containing LP-22 phage suspended in 1% peptone were submerged in a 60 C water bath. Samples were removed periodically

TABLE 2. *Sensitivities of the parent strain, the ts mutants, and two revertants to phage in the supernatant fluids of broth cultures of the mutants*

B. cereus test strain	Strain producing supernatant material					
	JS22	JS22-C[a]	JS23	JS25	JS26	JS29
T try$^-$	+[b]	–	+	–	+	–
JS22	–	–	–	–	–	–
JS22-C	++++	–	+++	+	+++	+
JS23	+++	–	++	++	+	+
JS25	++++	–	++++	+	++	+
JS26	++++	–	++++	+	+++	+
JS29	++++	–	++++	+	+++	+
JS22-R1	+	–	+	–	+	–
JS22-R2	+	–	+	–	+	–
JS23-R1	+	–	+	–	+	–

[a] JS22-C represents results for all five cured strains tested.

[b] –, No lysis; +, barely detectable lysis; ++++, confluent lysis.

and titrated by standard plaque assay technique. The experiment was also done at 70 C to determine whether the phage were able to survive the heat shock treatment of the spores. The results (Fig. 2) show that the phage was rapidly inactivated during the first 5 min and that a heat-resistant fraction of the phage was inactivated at a much slower rate. Similar results have been reported for other *Bacillus* phages (11).

Comparison of the plaque-forming phage produced by strain JS22 with phage CP-53. The LP-22 phage (Fig. 1A) is the predominant type found in JS22 and is absent from the cured strains of that mutant. Because the morphology of phage LP-22 is very similar to that of phage CP-53, the two viruses were compared with respect to host ranges, plaque morphology, antigenic similarities, and their sensitivities to ultraviolet light to determine whether they were identical.

No plaques were observed when phage LP-22 was added to soft agar overlays seeded with *B. cereus* ATCC6464 UM4, a strain sensitive to phage CP-53. Similarly, phage CP-53 failed to form plaques when added to lawns of the cured stocks of strain JS22. On sensitive hosts, CP-53 plaques were larger and more regular in shape than those of phage LP-22.

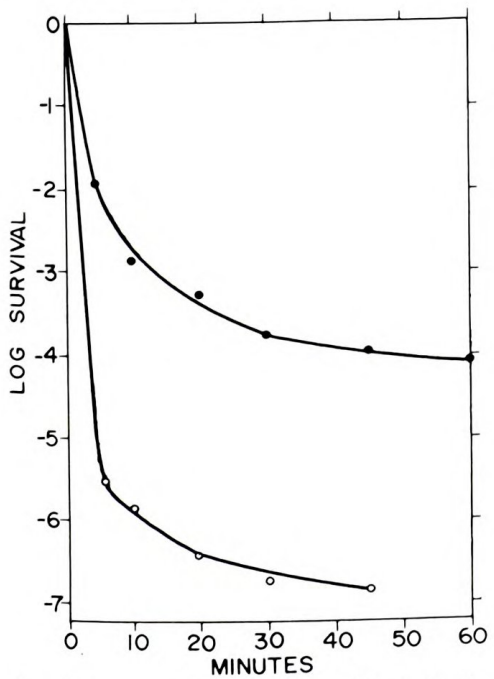

FIG. 2. *Time course of inactivation of Bacillus cereus phage LP-22 at 60 and 70 C in 1% peptone. The phage suspensions were submerged in a water bath at the desired temperature, and samples were removed periodically and assayed on lawns of JS22-C. Symbols:* ●, *60 C;* ○, *70 C.*

Antisera specific for each phage were tested for their abilities to inactivate each phage. The results (Table 3) show that each antiserum was specific for the phage against which it was prepared with only a slight cross-reaction between CP-53 and antiserum against LP-22 phage. Although the K values for the antisera were not determined, it is obvious that the two phages are different antigenically. LP-22 and CP-53 phages were similar in their sensitivities to ultraviolet light (Fig. 3).

Host range. A number of strains of *B. cereus* have been tested for their sensitivity to LP-22 phage and to the apparently defective phage produced by the cured strains. In addition, several other species of *Bacillus* have been tested for sensitivity to the defective phage, since these particles sometimes exhibit bacteriocin-like killing properties against other species (3). Strains tested include 16 strains of *B. cereus*, *B. megaterium* (KM, 19213), *B. subtilis* (168, Wb 746), *B. licheniformis* A2, *B. polymyxa*, and *B. thuringiensis*; the only strains sensitive to the plaque-forming phage have been mutants of *B. cereus* T and no strain has been sensitive to the "defective" phage.

Identification of the nucleic acid. LP-22 phage were fixed on glass slides as described above. When illuminated with ultraviolet light, all slides except the one treated with DNase yielded a green fluorescence. The slide treated with DNase failed to fluoresce. The green fluorescence indicated that the phage nucleic acid was double stranded, and loss of fluorescence on treatment with DNase but not RNase indicated that it was DNA (2).

Phage production by JS22 and by the parent strain. The time course of phage release was measured in broth cultures of JS22 and of the parent strain at both restrictive and permissive temperatures. CH medium was used to measure phage production by the mutant under the optimal conditions for sporulation at the permissive temperature. All cultures were inoculated with 3×10^6 heat-shocked spores per ml, which contained no detectable free phage. Figures 4A and B present typical experiments in which the appearance of phage was measured in supernatant fluids of cultures of strain JS22 and the parent strain grown at 37 C. During early exponential growth, the mutant produced 100 times more phage than the parent strain. The difference in the rate of phage production by the two strains became even greater in late exponential and stationary phases of growth. The

TABLE 3. *Specificity of antisera for CP-53 and phage from JS22*

Phage	PFU/ml after treatment with:		
	Preimmune serum	Anti-JS22 phage serum	Anti-CP-53 serum
JS22	4.5×10^8	0	8.9×10^5
CP-53	2.0×10^7	2.0×10^7	3.3×10^5

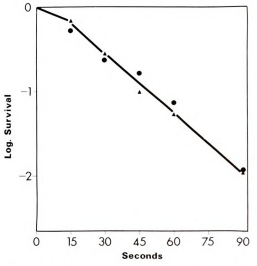

FIG. 3. *Inactivation of phages LP-22 and CP-53 by ultraviolet light. Petri dishes containing 10 ml of phage suspension in phosphate buffer were placed on a magnetic stirrer 40 cm from the source of ultraviolet light. Samples were taken at intervals and assayed on lawns of JS22-C and UM4. Symbols:* ▲, *LP-22,* ●, *CP-53.*

maximal number of phage produced by the mutant was approximately 6×10^5 times greater than the maximal number produced by the parent strain. When the same experiment was performed at 26 C (Fig. 4C and D) the mutant exhibited a slightly reduced capacity for phage production. The maximal number of phage produced by the mutant at 26 C was approximately 2.5×10^5 times greater than the maximal number produced by the parent strain. At both temperatures, over 99% of the total phage present in cultures of the mutants were not released until after the end of exponential growth.

Phage production has been measured in one of the revertants of JS22. The results at both temperatures were very similar to those obtained with the parent strain. Attempts to induce phage by treatment of the parent strain with ultraviolet light, at the dosage which allowed 37% of the cells to survive, failed to yield any increase in phage titers. Changing the dosage of ultraviolet light had no effect on phage production.

Attempts to measure the single-step growth curve for this phage with exponentially growing cells of strain JS22-C have not been successful. No bursts were observed even after 3 h of incubation at 37 C.

Stability of the phage-host relationship. Serial subculture of single cell lines has yielded cured derivatives of strains JS22, JS25, and JS26, but not of the parent strain. This indicates that the phage probably forms a less stable relationship with the mutants that with the parent strain.

Attempts were made to determine the percentage of spores of strain JS22 and parent strain which carry the phage. In a typical experiment, colonies derived from spores were grown on plates at 37 C for 18 h, overlaid with soft agar seeded with JS22-C cells, and incubated for an additional 12 h. The colonies that produced phage caused a localized clearing of the indicator strain. These were counted and the plates were then incubated for another 12 h, after which the colonies producing phage were again counted. In no case was it possible to detect phage production by the parent strain. Many of the colonies of strain JS22 did not produce detectable titers of phage until they were over 30-h old. The proportion of spores of JS22 which produced detectable phage was 36% before heat shocking and 35% after heat shocking at 70 C for 30 min. Since the lawns of indicator cells began to lyse spontaneously after 24 h (42-h colony age), it was not possible to determine the percentage of colonies capable of inducing phage after longer incubation periods. Thus, the data probably underestimate the fraction of spores carrying phage. However, the results do indicate that the phage was carried by the spores in a heat-stable form.

DISCUSSION

The five ts mutants appear to be similar in their sporulation properties. However, it is unlikely that the ts sporulation mutants are identical since they were obtained from three separate mutagenesis treatments and they exhibit differences with respect to their abilities to produce phage (Table 2). Lawns of strain JS22 do not produce spontaneous discrete plaques and are apparently insensitive to superinfection. Strain JS29 produces large, clear plaques spontaneously and upon infection with LP-22 phage. Presumptive evidence suggests that they all lack a functional tricarboxylic acid cycle and are blocked very early in sporulation. Further

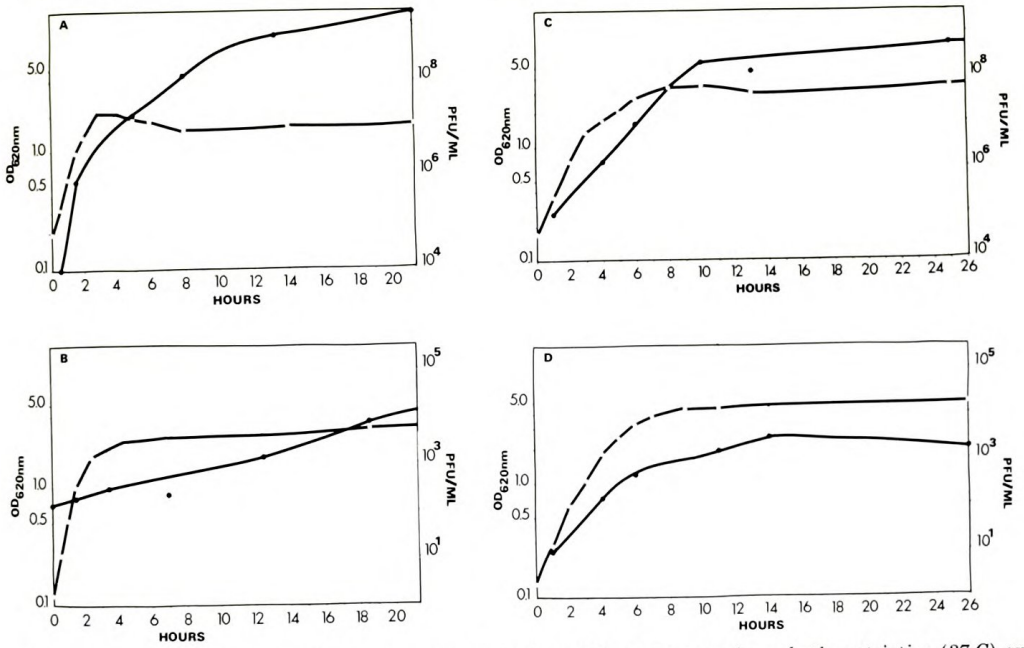

FIG. 4. *Growth and phage production of mutant JS22 and the parent strain at both restrictive (37 C) and permissive (26 C) temperatures. Growth was measured by following changes in optical density (620 nm), and PFU per milliliter were assayed on lawns of JS22-C. (A) Mutant JS22 at 37 C. (B) B. cereus try⁻ at 37 C. (C) Mutant JS22 at 26 C. (D) B. cereus try⁻ at 26 C. Symbols: ○, optical density; ●, PFU/ml.*

evidence for blockage at the beginning of sporulation was obtained from observations of thin sections of strain JS22 under the electron microscope.

The production of high titers of phage by strain JS22 appears to be related in some way to the single-step mutation affecting its ability to sporulate, since single-step revertants, selected for their ability to sporulate normally at the restrictive temperature, did not retain the ability to produce high titers of LP-22 phage or the sensitivity to LP-22 phage found in the mutants. However, the relationship between LP-22 production and sporulation does not appear to be direct, since the efficiency of phage production, unlike that of sporulation, is not highly dependent on temperature.

The association of phage production with impaired ability to sporulate has been observed in other species of *Bacilli*. Thorne noted that *B. subtilis* W-23-S^r produced very low titers of the phage SP-10 when grown under conditions in which high yields of spores were obtained and very high titers under conditions in which few spores were obtained (14). Ito and Spizizen observed that *B. subtilis* phage $\phi 2$, which cannot multiply in *B. subtilis* 168, was able to multiply in mutants of *B. subtilis* 168 that were defective in early spore functions (9). They suggest that an alteration of the ribonucleic acid polymerase in the mutants or an alteration of unique membrane components involved in phage restriction may be responsible. These possibilities also exist for our system, since sporulation is somewhat impaired in strain JS22 even at the permissive temperature (Table 1).

The results of several experiments suggest that the phages produced by the ts mutants are of several morphologies, at least one which forms plaques (LP-22) and another, produced by strains cured of the lytic phage, which is defective. Although the heads of these phages are all of the same size and shape and the end plate structures are identical, there are differences in the size and morphology of the tails. The tails of the presumed plaque formers are 11.5-nm wide and appear to be typical noncontractile tails. The tails of the other types are 9.5-nm wide, are variable in length, and have a variable number of platelike structures attached perpendicular to the tail axis. Some of these platelike structures have fibers extending from them.

The phages with the abnormal tail structures do not appear to be morphological variants of LP-22. All strains of the mutants which appear to be cured of the plaque-forming phage yield

the unusual phage. These strains were judged to be cured by their inability to lyse sensitive lawns on agar overlay plates and their sensitivity to the plaque-forming phage. All phage detected in electron microscope observations of supernatant solutions (lysates) of the cured strains have been the defective type. At this time it is not known whether production of this second phage is related to the ts sporulation mutations.

Goldberg and Gollakota (6) and Gollakota and Halvorson (1960, Bacteriol. Proc., p. 86) have isolated and studied a phage from *B. cereus* T similar to LP-22 phage with respect to its release into the medium during the stationary growth phase. However, the properties of the phage (6), tail measuring 400 nm in length and 25 nm in diameter and its sensitivity to chloroform, preclude the possibility of its identity with LP-22. The nonidentity of phage LP-22 with phage CP-53 has been established by comparing the host specificities, antigenic cross-reactivities and plaque morphologies of these two phages.

It appears that LP-22 phage is carried by the host either in a lysogenic state or in a carrier state similar to that of SP-10 phage in *B. subtilis* (10). This is demonstrated in the parent strain by its apparent immunity to infection and by the spontaneous production of low titers of phage in broth cultures of that strain. Although the mutants produce higher titers of the phage and appear to have become sensitive to it, the failure of the phage to cause confluent lysis and the production by the phage of small turbid plaques on lawns of the mutants indicate that the virus-host relationship is still reasonably stable in the mutants.

It is not possible to establish, with the present data, whether the stable relationship between the phage and the host is due to the establishment of true lysogeny or to the establishment of a pseudolysogenic or carrier state. The isolations of cured derivatives of strain JS22 by serial subculture of single cell lines suggests that the phage-cell relationship in the ts mutants is that of pseudolysogeny. On the other hand, the failure of serial subculture to produce cured parent strains suggests in this case that the more stable phage-cell relationship may be one of true lysogeny.

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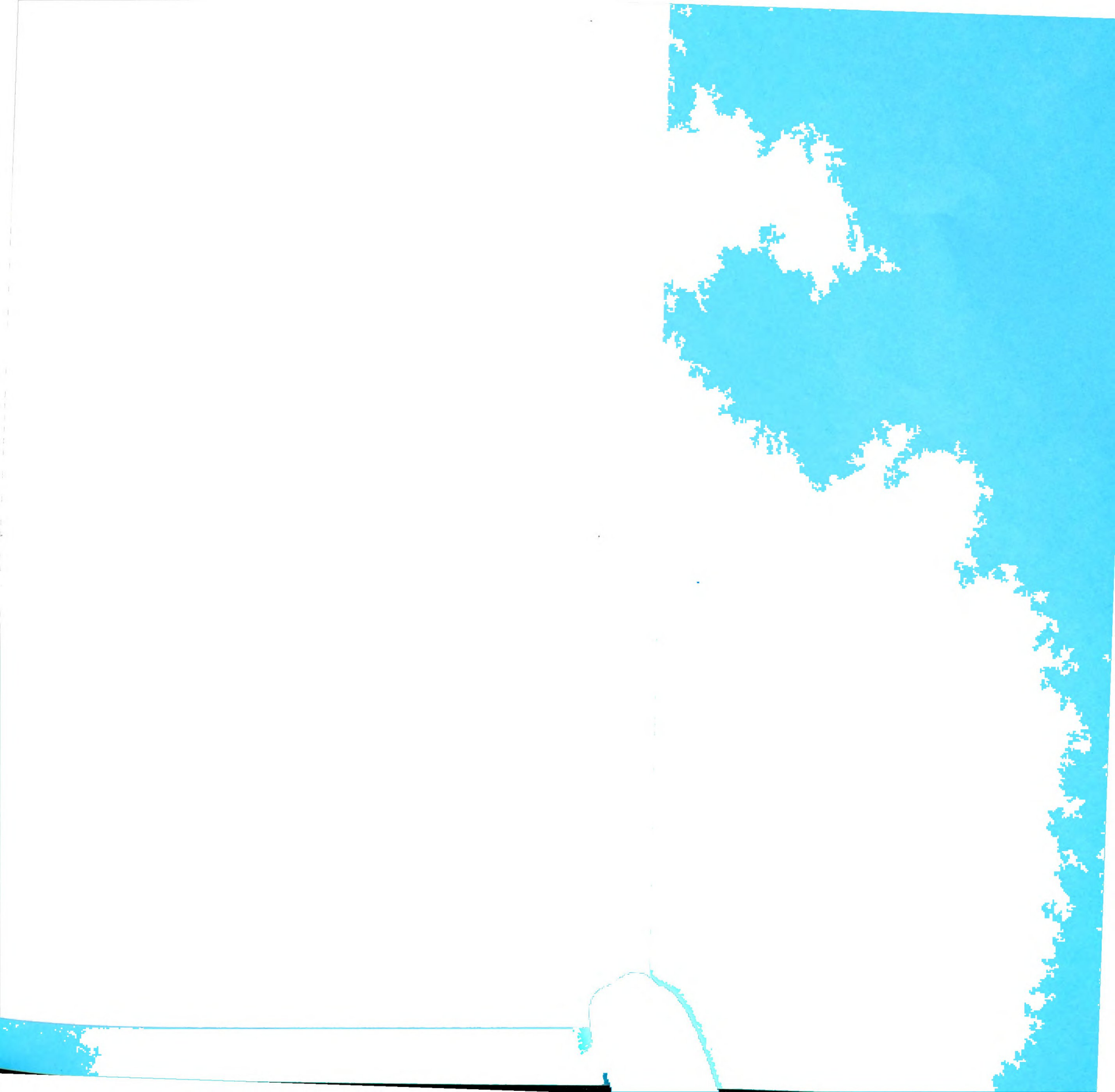

ARTICLE 2

PRELIMINARY INVESTIGATION OF A TEMPERATURE-SENSITIVE SPORULATION MUTANT OF *BACILLUS CEREUS* T

By

G. N. Stelma Jr. and H. L. Sadoff

(Manuscript in preparation)

ABSTRACT

Growth of temperature-sensitive mutant JS22-C occurs normally at the restrictive temperature (37 C), but sporulation is blocked at stage 0. The production of extracellular and intracellular proteases and of alkaline phosphatase occurs at 37 C, but the expression of a functional tricarboxylic acid cycle does not. At the permissive temperature (26 C), the mutant sporulates at a slightly lower frequency (60%) and at a slower rate than the parent strain at 26 C. The oxidation of organic acids that accumulate during vegetative growth begins at T_o in cultures of the parent strain but is delayed until about T_3 in cultures of the mutant. Later events in sporulation are also delayed by about 3 hours. Experiments, in which the temperature of growth was shifted from 37 C to 26 C or from 26 C to 37 C at various times, have shown that the temperature-sensitive event begins approximately an hour after the end of exponential growth and ends when the cells reach the end of stage II (septum formation). The absence of a functional tricarboxylic acid cycle in cells of the mutant grown at 37 C or

shifted from 26 C to 37 C before T_1 does not appear to be due to a lesion in one of the structural genes of the tricarboxylic acid cycle but is more likely due to the inability of the cells to derepress the synthesis of some of the enzymes of that cycle.

INTRODUCTION

Bacterial sporulation occurs as a precisely ordered sequence of events which result in the formation of a complex intracellular structure, the bacterial endospore (10). The sequence of events which lead to the formation of an endospore differs from the sequence that occurs during normal cell division, therefore sporulation is considered to be a form of cellular differentiation and is being studied as a model to gain an understanding of principles applicable to cellular differentiation in higher systems (10, 20, 27).

Temperature-sensitive (ts) asporogenous mutants are valuable tools for studying gene function during sporulation. They can be used to obtain valuable information which totally negative mutants are unable to provide. Single point ts mutants enable one to determine whether the mutation resides in a structural gene or a control gene; they also permit one to control the phenotypic expression of a single gene product by shifting the temperature of the cultures. From these temperature-shift experiments, the time period during sporulation can

be determined in which the function of the mutated gene is indispensible (4, 15).

Recently several groups have studied ts mutants which were blocked at various stages of sporulation when grown at restrictive temperatures. Szulmajster and co-workers (8, 35) isolated a mutant of B. subtilis which grew normally at 30 C and at 42 C but was blocked at stage 0 of sporulation at 42 C. In vitro protein synthesis studies revealed that this mutant had a low protein synthesizing capacity at 42 C and led to the discovery of a nondialyzable inhibitor which affected amino acylation of phenylalanyl tRNA (8). The full significance of this observation is still not known. Hitchins and Sadoff (14, 15) have studied a mutant of B. megaterium which, when grown at 39 C, is blocked in some function essential both to the formation of a normal cell division septum and to the formation of a forespore septum. The nature of the product of the mutant gene has not been determined. Leighton and his co-workers have used a more direct approach. They have isolated various ts mutants of B. subtilis affecting specific enzymes which they believed were involved in sporulation (21, 22, 23, 29, 30). One series of ts mutants isolated by Leighton (21) include rifampin-resistant RNA polymerase mutants which are affected at uniquely different time periods during sporulation. One of them, ts-14, produces several

of the products involved in the early stages of sporulation but is affected in forespore membrane synthesis and engulfment and synthesis of alkaline phosphatase (21, 30).

This report describes properties of a ts stage 0 mutant of B. cereus, JS22, where isolation was the subject of a previous communication (34). Upon growth at 37 C, this organism became sensitive to lysis by a bacteriophage which it carried. We now report some further characteristics of JS22-C, a derivative of mutant JS22 which has been cured of the phage genome.

MATERIALS AND METHODS

Bacterial strains and cultivation. The parent strain used in this study was a tryptophan auxotroph of Bacillus cereus T, designated in our laboratory isolate JS1. The mutant, JS22-C, was a temperature-sensitive sporulation mutant which had become spontaneously cured of its phage, designated LP22. The permissive and restrictive temperature for sporulation of JS22-C were 26 and 37 C respectively. JS22-C is able to sporulate at a higher frequency than JS22 when grown at the permissive temperature. The procedures used for the isolation of the mutant and for the production and storage of stock spore suspensions have been described in an earlier publication (34).

The procedure used for growing cultures of the parent strain and the mutant were the same in all experiments. Approximately 1×10^8 spores were heat-shocked (70 C for 30 min.) and inoculated into flasks (500 ml) containing 50 ml nutrient broth. The flasks were incubated at either 26 or 37 C, depending on the particular experiment in a New Brunswick model G76 water bath shaker at a speed of 240 rpm. When these cultures

reached mid-exponential growth, 5 x 10^8 cells were removed and used to inoculate flasks (500 ml) containing 100 ml modified G medium (13) supplemented with 20 ug of L tryptophan per ml. These cultures were grown under the same conditions used for the nutrient broth starter cultures. Growth was followed by measuring change in turbidity (OD_{620}) on a Bausch and Lomb Spectronic 20 spectrophotometer.

Electron microscopy. Cells were collected on 0.45 μm membrane filters (Gelman), washed with cold 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and suspended in 1% Noble agar (2). After solidification the agar was diced and stored in the glutaraldehyde fixative at 4 C for 48 h (28). The pieces of agar containing the fixed cells were rinsed five times in cold 0.1 M phosphate buffer (pH 7.2) and postfixed overnight at room temperature in Kellenberger's buffer containing 1% W/V osmic acid (19). The specimens were then stained 2 h in buffered uranyl acetate.

The fixed cells were dehydrated in a series of alcohol solutions followed by propylene oxide and were embedded in epon as described by Luft (25). Thin sections were cut on a diamond knife mounted on an ultratome III (LKB Produkter, Stockholm, Sweden). These were then poststained for 30 min. with 2% aqueous uranyl acetate (37) and for 10 min. with lead citrate (5).

Observations and photographs were made on a Hitachi Hu-11 electron microscope or on a Phillips EM 300.

Enzyme assays. Protease activity was measured by the rate of release of acid-soluble chromophoric groups from azo-albumin (Sigma) as described by Millet (26) except the addition of 0.5 N NaOH was omitted and the absorbance was measured at 340 nm rather than 440 nm. All protease assays were performed at 37 C. Alkaline phosphatase activity was measured using p-nitrophenyl-phosphate (Nutritional Biochemicals) as a substrate by the method of Warren (36). The oxygen uptake of the culture at various times in sporulation was used as an index of the activity of the tricarboxylic acid cycle and was measured by monitoring the reduction of a dye, 2, 6-dichlorophenol indolphenol (DCPIP) in cell suspensions. Assay mixtures consisted of 0.2 ml of cell culture, 0.3 ml KCN (10^{-3}M) 0.2 ml DCPIC (2×10^{-6}M) and 2.8 ml of cell-free culture fluid (cells removed by centrifugation). All assays were done at 37 C. Reduction of the DCPIP was measured at 540 nm (molar absorbance coefficient of 1.6×10^4 cm^2/mole) on a Perkin Elmer double beam spectrophotometer and recorded on a Sargent recorder model SR.

Temperature-shift experiments. Cultures were grown in modified G medium as described above. In the shift down experiments, 5.0 ml samples were removed from a culture at 37 C and inoculated into sterile flasks

(125 ml). These were immediately placed on a 26 C shaker. At T_o (the end of exponential growth) a 5.0 ml subculture of the wildtype was placed on the 26 C shaker to serve as a control, and a 5.0 ml subculture of the mutant was taken and incubated at 37 C, as a second control. At T_{24}, the subcultures were subjected to heating at 70 C for 30 min. Dilutions of the heated spore suspensions were spread on nutrient agar plates, and the colonies were counted after overnight incubation. The shift up experiments were done in a similar manner except that the subcultures were left on the shaker until T_{30}.

RESULTS

Growth and sporulation at 37 C. In order to determine whether the gene affected by the ts lesion in mutant JS22-C was critical for sporulation specifically or if it also affected vegetative cell processes, the growth rates of the mutant and parent strain were compared at the restrictive temperature (37 C). Typical growth curves for both strains are shown in Figure 1. In five separate experiments the generation time for the parent strain varied from 31.6 min. to 34.8 min. with an average generation time of 33 min. Under the same conditions, the generation time for the mutant varied from 33.4 min. to 36.6 min. with an average generation time of 34.7 min. These results indicated that growth at 37 C did not have any significant effect on the vegetative metabolism or on the cell division process of JS22-C. Elevating the temperature of growth to 45 C still failed to produce a difference in growth rates. Vegetative cells of JS22-C grown at 37 C were identical to those of the parent strain in appearance when viewed by phase contrast or electron microscopy.

When cultures of Bacillus species are grown in media containing glucose, the pH of the cultures drops from 7.0 to approximately 5.5 because of the accumulation of organic acids. At the end of exponential growth (T_o) with the exhaustion of glucose, the synthesis of the enzymes of the tricarboxylic acid cycle is derepressed and the organic acids are oxidized with a subsequent use in the pH of the medium (3, 9, 11).

To aid in the determination of T_o, the pH of cultures of the parent strain and the mutant was measured at various times during growth and in stationary cultures (Figure 1). The pH of the cultures of the parent strain dropped steadily during exponential growth until it reached a value of approximately 5.2 at T_o. By T_1 an increase in pH became apparent indicating that the tricarboxylic acid cycle enzymes were derepressed between T_o and T_1. By T_6, when refractile forespores were beginning to appear in the parent strain, the pH had usually increased to values between 6.6 and 7.0. The pH cultures of the mutant grown at 37 C dropped to 4.9 at T_o and never increased, suggesting that the tricarboxylic acid cycle was not functional. The lower cell density in stationary cultures of the mutant, Fig. 1, is a characteristic response.

The production of exoproteases, an event associated with the end of exponential growth (24, 31),

was measured in the parent and the mutant strains at 37 C. The mutant produced normal levels of exoprotease, and the kinetics of exoprotease production by the mutant and the parent strain were identical (Figure 1). Studies with ethylene diamine tetra-acetic acid (EDTA), which inhibits the metal requiring protease, and phenylmethyl sulfonyl floride (PMSF), which inhibits the serine protease, revealed that both types of protease were present in supernatant fluids of JS22-C cultures grown at 37 C. Approximately 95% of the activity of the exoprotease was inhibited by EDTA in both the parent and mutant strains. Alkaline phophatase, another exoenzyme produced during sporulation, was present at the same level in culture supernatant fluids of the parent and mutant strains of B. cereus. Recent observations that the extracellular and intracellular serine proteases of B. subtilis have different electrophoretic mobilities (12) and that the regulation of the exoprotease and endoprotease is not necessarily coupled in B. cereus (Cheng, and Aronson, Bacteriol. Proc. P. 39, 1974) prompted us to examine endoprotease activity in the mutant. Cells of the parent strain and of JS22-C were harvested at T_1, washed twice in 0.2 M tris-HCl (pH 7.2), and broken by sonication. The crude extracts were assayed for proteolytic activity and both metal protease and serine protease activities were detected in similar levels in the mutant and wild

type organisms. In each strain only 10 to 12% of the total proteolytic activity in the crude extracts was inhibited by EDTA.

Refractile forespores were first detected microscopically at T_6 in cultures of the parent strain grown at 37 C. By T_8, all of the cells contained refractile forespores. Refractile forespores did not develop in cultures of the mutant JS22-C at 37 C. The parent strain produced an average of 7×10^8 heat-stable (70 C, 30 min.) spores per ml but the mutant only produced between 1×10^3 and 5×10^3 heat-stable spores per ml in modified G medium at 37 C.

Thin sections of mutant cells, grown at 37 C for the time period required for the parent strain to form mature spores, were examined under the electron microscope. No evidence for the formation of an axial filament or a forespore septum was seen in any of the cells; this indicated that the cells were blocked at stage 0. A typical longitudinal section is shown in Figure 2.

Growth and sporulation at 26 C. When the mutant was grown at the permissive temperature (26 C), the vegetative growth rate and production of exoprotease paralleled that of the parent strain (Figure 3). The average generation times for the parent strain and mutant were 48.5 min. and 47.2 min. respectively. Although the

mutant was able to produce spores with normal heat stability at 26 C, the sporulation process was somewhat impaired. The increase in the pH of the culture fluid, which is indicative of tricarboxylic acid cycle activity, was observed in cultures of the parent strain immediately after T_o, but in cultures of the mutant this increase was not seen until T_3 (Figure 3). Later events in sporulation also appeared to be delayed with respect to the time of expression. The development of refractility occurred between T_{10} and T_{16} in cultures of the parent strain and between T_{13} and T_{19} in cultures of the mutant (Figure 3). Cultures of the parent strain produced approximately 6×10^8 heat-stable (70 C, 30 min.) spores per ml, while those of the mutant produced 3.5×10^8 heat-stable spores per ml.

Temperature-sensitive period. Temperature shift experiments were performed with the mutant strain JS22-C to determine the time period in which the ts gene functioned and to determine whether the loss in sporulation capacity at 37 C was reversible. A similar experiment was performed using the parent strain to determine whether the temperature shift had any effect on its capacity to sporulate. The results of two typical shift down-experiments with mutant JS22-C are shown in Figure 4. The pH changes were identical in both experiments. Subcultures shifted from 37 C to 26 C at T_1 or earlier were

able to recover completely and produce approximately the same number of heat-stable spores per ml as cultures maintained at 26 C. If subcultures were shifted after $T_{0.5}$ the sporulation capacity of the mutant decreased progressively. If the shift was made at T_3, less than one percent of the cells were able to sporulate and, if it was made at T_4 or later, the cells were unable to sporulate. Measurements of the final pH of the subcultures revealed that the cells were unable to oxidize organic acids in the medium if the shifts were made at T_4 or later.

In similar experiments subcultures of the ts mutant were shifted up from 26 C to 37 C (Figure 5). Those shifted at T_4 or earlier sporulated at very low frequencies. Sporulation capacity increased significantly if the shift was made at T_5 and reached near maximal values if the shift was made at T_6 suggesting that the ts gene product was required until T_6. Thin sections of JS22-C cells removed from 26 C cultures at T_6 were examined under the electron microscope. All cells had formed completed or nearly completed forespore septa, but none had begun the engulfment process. These observations indicate that the cells were at the end of Stage II (forespore septum formation) when the function of the ts gene was completed. The appearance of a typical cell at T_6 is shown in Figure 6.

Measurements of the pH in the culture fluids at T_{30} revealed that all subcultures shifted after T_1 had gained the capacity to derepress the enzymes of the tricarboxylic acid cycle; while those shifted before T_1 never gained that capacity. These data suggest that the expression of that gene was necessary before the tricarboxylic acid cycle could become functional. Although expression of the ts gene until T_1 was sufficient to derepress the tricarboxylic acid cycle enzymes, continued function of this gene until T_6 was necessary before sporulation could occur at near normal frequencies (Figure 5).

Effect of temperature shift on tricarboxylic acid cycle activity. Since mutants of Bacillus subtilis blocked in the inability to produce a functional tricarboxylic acid cycle have been observed to be impaired in their ability to sporulate (3, 6, 9), the possibility that the genetic lesion in JS22-C resided in a structural gene for one of the tricarboxylic cycle enzymes was investigated. The extent of oxidative metabolism and thus, indirectly, the activity of the tricarboxylic acid cycle was determined by dye reduction studies. Rates of oxidation determined in this manner for the parent strain were similar to those previously reported by Gollakota and Halvorson for B. cereus (7). Cultures of the parent strain and mutant were shifted from 26 to 37 C at the

times when the rise in pH was first detected in the culture medium (Figure 3). Activities of the tricarboxylic acid cycle were expected to the highest at these times. When JS22-C cultures were shifted at T_3, the rate of oxygen consumption 30 min. after the shift had increased significantly over the initial rate, indicating that the activity of the tricarboxylic acid cycle had increased (Table 1). One hour after the shift the rate of oxygen consumption had decreased somewhat but was still higher than the initial values. The maximal rate found at $T_{3.5}$ was almost as high as the maximal rate attained by the parent strain at T_1. If the temperature-sensitive protein of the mutant were one of the tricarboxylic acid cycle enzymes a significant decrease in the activity of the tricarboxylic acid cycle would have occurred rather than the increase that was observed. Cultures of the mutant shifted from 26 C to 37 C at T_3, and shifted back to 26 C at T_4, produced 40% fewer heat-stable spores than control cultures of JS22-C which were left at 26 C. Sporulation was thus much more sensitive to the temperature shift during this interval than was the activity of the tricarboxylic acid cycle.

Cultures of the mutant shifted to 37 C at T_1 had no detectable oxidative capacity at the time of the shift (Table 1). At T_2 significant activity was detected although this activity was low compared to the activities

observed in the parent strain. This activity remained about the same at T_3 and declined sharply at T_4. The initial increase in oxidative metabolism, coupled with the slow decrease after the shift to 37 C, is inconsistent with the supposition that the temperature-sensitive protein was one of the tricarboxylic acid cycle enzymes.

DISCUSSION

Growth studies of mutant JS22-C at 37 C have shown that the function of the ts gene is not essential for normal vegetative growth or for the production of proteases and alkaline phosphatase at the onset of sporulation. However, the function of the ts gene appears to be essential for the expression of a functional tricarboxylic acid cycle and for the cells to undergo any of the morphological changes associated with sporulation. Cells of JS22-C maintained at 37 C throughout growth were blocked at stage 0 of sporulation.

Growth studies of the ts mutant at 26 C revealed that it was slightly impaired in its ability to sporulate at that temperature. The mutant at 26 C produced only 60% as many spores as cultures of the parent strain. At the permissive temperature the number of heat-resistant spores per ml produced was 10^5 fold greater than the number produced at 37 C, the restrictive temperature. At the permissive temperature, the expression of the tricarboxylic acid cycle enzymes and the development of refractility did not occur in the mutant cultures

until about three hours after they had occurred in the parent strain. However, the kinetics of exoprotease production were the same in both mutant and parent strain. These observations are in accord with the data obtained in studies at the restrictive temperature showing that protease production is not impaired by the mutation. Those cellular activities leading to sporulation which are blocked at 37 C appear to be impaired in JS22-C at 26 C. Those not blocked at the restrictive temperature are normal at the permissive temperature.

Experiments in which cultures of the mutant were shifted from 37 C to 26 C (Figure 4) or from 26 C to 37 C (Figure 5) at various times during the growth cycle have shown that the temperature-sensitive event occurs very early in the sporulation sequence, those cells of JS22-C maintained at 37 C for four or more hours after the end of exponential growth cannot recover the ability to produce spores if they are shifted to 26 C. Morphological studies have shown that the expression of the ts gene begins at the sporulation stage preceding the formation of the axial filament (Figure 2) and ends when the forespore septum has been completed (Figure 6).

Since the tricarboxylic acid cycle is not functional in the mutant at 37 C, and a block in any one of the enzymes of that cycle has been shown to impair the ability of mutants to sporulate (3, 6), the

temperature-sensitivity of the tricarboxylic acid cycle of JS22-C was investigated. The results of these studies (Table 1) indicated that the temperature-sensitive lesion had not occurred in any of the structural genes which code for the tricarboxylic acid cycle enzymes or for any of the electron transport components ultimately reacting with the dye. The initial increase followed by a slow decrease in oxidative metabolism after the mutant cultures were shifted from 26 C to 37 C would not be expected if one of the many proteins involved were subject to decay at the higher temperature. If any one of the enzymes involved in oxidation of organic acids were subject to decay at a very slow rate at 37 C, some oxidative metabolism should have been detectable in cultures of the mutant maintained at 37 C throughout growth. However, cultures maintained at 37 C and assayed several times between T_o and T_5 did not produce detectable dye reduction (unpublished results). The delay in expression of oxidative metabolism (TCA cycle activity) in cultures grown at 26 C is not consistent with the supposition that the ts mutation affects the activity of one of the individual enzymes of that cycle. The hypothesis that the lesion has affected a control gene which must be expressed before derepression of the tricarboxylic acid cycle can occur is more consistent with our data.

Cultures of the mutant growing at 26 C have undergone derepression of the tricarboxylic acid cycle at T_4 (Figure 3, Table 1); but these cultures are not able to produce a significant number of spores if they are shifted to 37 C. Thin sections of cells removed from 26 C cultures and fixed at T_4 have been compared with thin sections of cells removed from cultures which were shifted from 26 C to 37 C at T_4. The cells which were shifted up were observed to be arrested at the stage of morphological development they had attained at the time of the shift (unpublished observations). These observations imply that the function of the ts gene is necessary for the expression of at least one sporulation-specific event that occurs after the tricarboxylic acid cycle has been derepressed.

The ts mutation which blocks the ability of this mutant to sporulate at 37 C also appears to be responsible for the induction of LP22, a bacteriophage carried by the parent strain in a latent form. Revertants of mutant JS22 selected for their ability to sporulate at 37 C concurrently lost the ability to induce the phage at high frequencies (34). Revertants of JS22-C which were able to sporulate at 37 C at high frequencies did not support plaque formation as well as JS22-C or as partial revertants which sporulated at frequencies of 1% or less. This inverse relationship between the ability of cured

strains to sporulate and their ability to support plaque formation further supports the hypothesis that the ts mutation somehow affects the stability of the phage-host relationship between LP-22 and B. cereus (34).

Studies of the P22-Salmonella typhimurium system have shown that the establishment of lysogeny or replication depends upon the level of cyclic adenosine 3', 5' - monophosphate (cAMP) in the host cells. Under conditions of strong catabolite repression, when the supply of energy and biosynthetic components is abundant and the concentration of cAMP is low, the phage multiplies and lyses the cell. When the supply of energy is deficient and the concentration of cAMP is high the phage lysogenizes the cell (16).

Although cAMP is apparently absent from cells of Bacillus (18, 33), some alternative signal must be used to indicate a depletion in energy levels since sporulation has been shown to be subject to catabolite repression (32) and dependent on the energy charge of the cell (17). If the lytic cycle of LP22, like that of P22, is dependent on the energy level of the host cell, a mutation which produces a state of strong catabolite repression could cause induction of phage to occur at an increased frequency and concomitantly prevent derepression of sporulation.

If the ts mutation in JS22 has affected the ability of the cells to overcome catabolite repression, it has affected a specific mechanism for release of catabolite repression rather than a generalized mechanism. This is evident from the ability of the mutant to synthesize normal levels of exoprotease at the restrictive temperature. Aronson et al. (1) have demonstrated the existence of closely related but distinct catabolic controls for extracellular protease production and for spore formation in Bacillus cereus. Furthermore, the parent and mutant strains undergo diauxic growth at 37 C in modified G-medium containing low levels of glucose supplemented with maltose or sucrose. Thus normal catabolite repression seems to function in the mutant JS22-C but the release of special catabolite repression associated with certain sporulation events seems to be blocked.

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TABLE 1. Oxygen consumption by cells of the parent strain and mutant JS22-C after shifting growth temperature from 26 C to 37 C

Sample	Oxygen consumption						
	T_o	$T_{o.5}$	T_1	T_2	T_3	$T_{3.5}$	T_4
parent strain shifted at T_o	1.8	7.8	8.8	8.3	-	-	-
mutant JS22-C shifted at T_3	-	-	-	-	5.6	8.4	7.0
mutant JS22-C shifted at T_1	-	-	0	3.0	2.7	-	1.0

Cultures of the parent strain and mutant JS22-C were grown in G medium at 26 C. At the times indicated, above the cultures were shifted to 37 C. Oxygen consumption was measured immediately and several times after the shift. Oxygen consumption was measured by measuring the rate of reduction of the dye 2, 6 dichlorophenol-indolphenol at 540 nm by whole cells. In the presence of KCN, the dye becomes the terminal electron acceptor. Two moles of the dye reduced are equivalent to a mole of oxygen consumed. Oxygen consumption is expressed in ul 0_2 consumed per 10^8 cells per h at STP.

FIGURE 1. Growth of the parent strain and the mutant at the restrictive temperature (37 C). Growth was monitored by measuring changes in OD_{620}. In order to determine T_o, the end of exponential growth and onset of sporulation, pH values were also measured. Total exoprotease activity and the development of refractility were also measured, these are expressed in terms of percent of the maximal values obtained. Time is expressed in terms of T_o Symbols: ● OD_{620} parent strain; O OD_{620} JS22-C; ■ pH parent strain; □ pH JS22-C; ▲ exoprotease activity parent strain; Δ exoprotease activity JS22-C; ⊖ refractility parent strain.

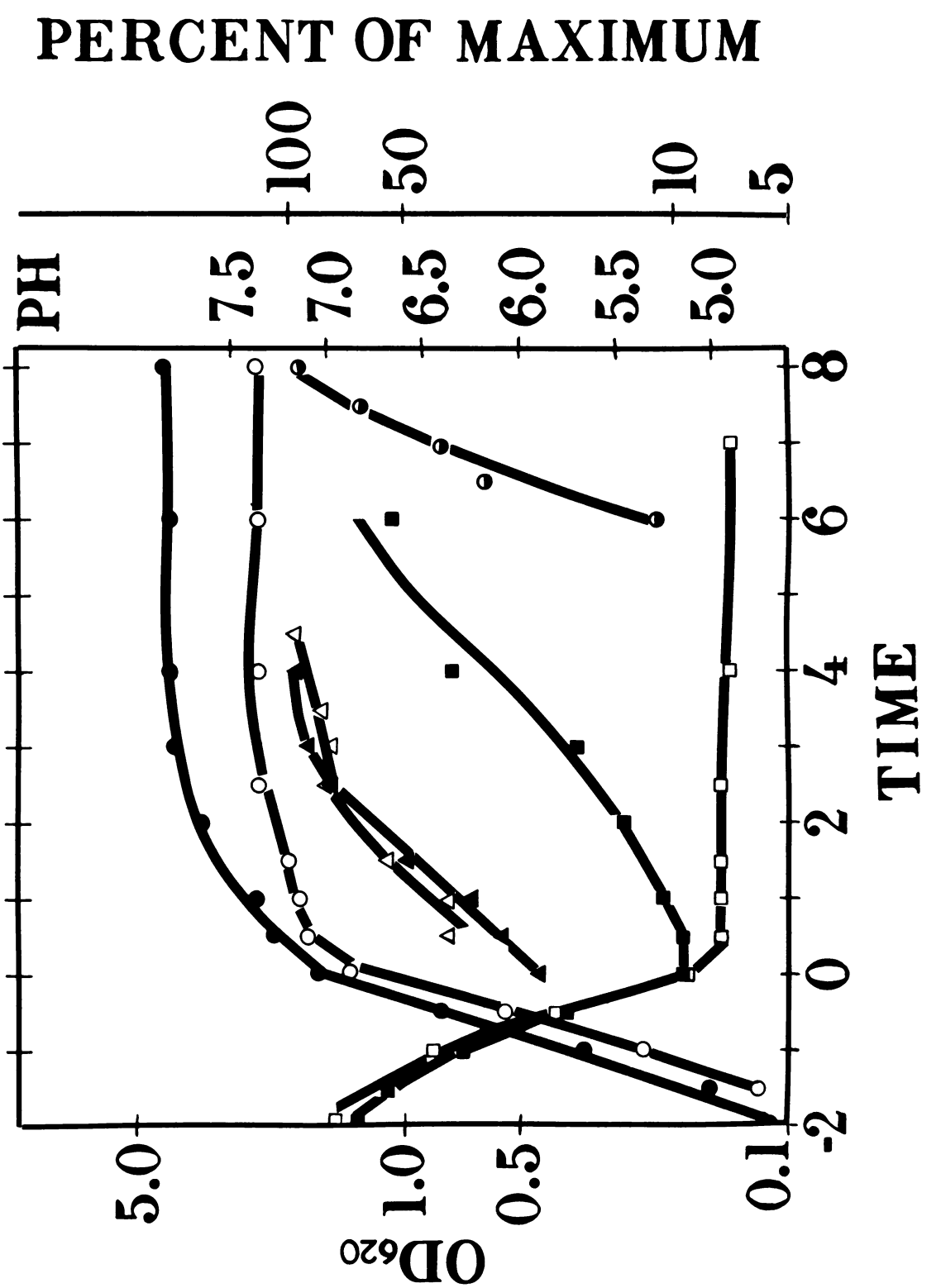

PERCENT OF MAXIMUM
100
50
10
5
PH
7.5
7.0
6.5
6.0
5.5
5.0
8
6
4
2
0
-2
TIME
5.0
1.0
0.5
0.1
OD620

FIGURE 2. Electron micrograph of a longitudinal section of the mutant after growth at 37 C for the time required for the parent strain to sporulate under the same conditions. The bar in the lower left-hand corner represents one micron.

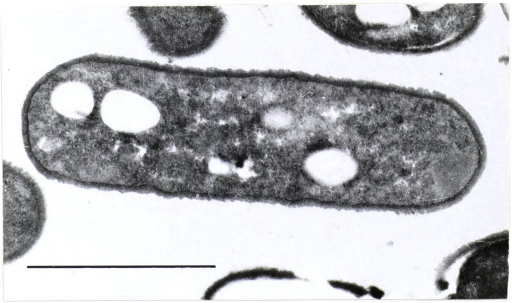


FIGURE 3. Growth of the parent strain and mutant at the permissive temperature (26 C). Growth was monitored by measuring changes in OD_{620}. Changes in pH, total exoprotease activity and the development of refractility were also measured. Exoprotease activity and refractility are expressed in terms of percent of the maximal values obtained. Time is expressed in terms of T_o, the end of exponential growth. Symbols: ● OD_{620} parent strain; O OD_{620} JS22-C; ■ pH parent strain; □ pH JS22-C; ▲ exoprotease activity parent strain; Δ exoprotease activity JS22-C; ◒ refractility parent strain; ◓ refractility JS22-C.

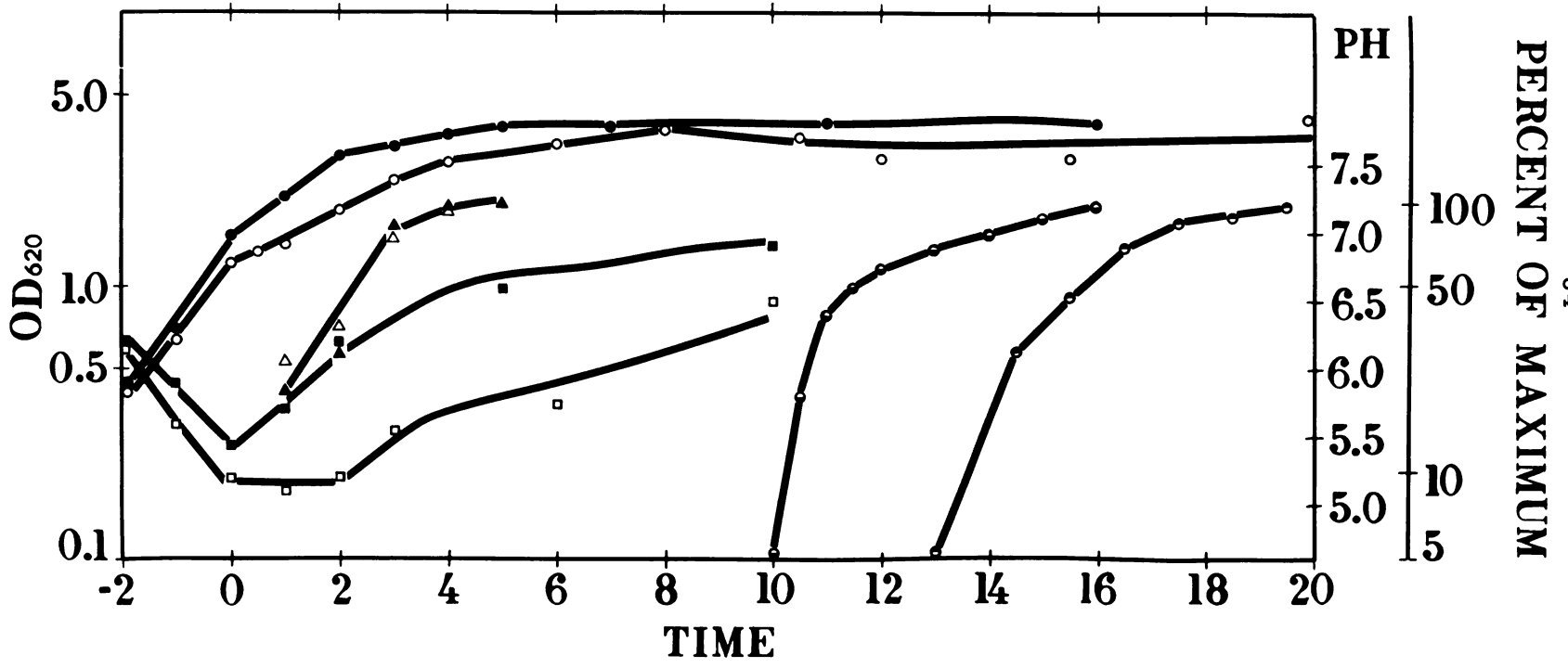

5.0
1.0
0.5
0.1
OD_{620}
-2
0
2
4
6
8
10
12
14
16
18
20
TIME
PH
7.5
7.0
6.5
6.0
5.5
5.0
100
50
10
5
PERCENT OF MAXIMUM

FIGURE 4. Sporulation of JS22-C cultures shifted-down from 37 C to 26 C at different times during the growth cycle. T_o designates the end of exponential growth. Heat-resistant spores were counted by plating the spore suspensions at T_{24} after heating at 70 C for 30 min. The pH was measured at T_{24}. The bar designated W^t represents the spore yield in cultures of the parent strain which were shifted to 26 C at T_o. The bar designated 37 C represents the spore yield in cultures of JS22-C maintained at 37 C throughout the course of the experiment. Symbols: ● heat-resistant spores, experiment 1; O heat-resistant spores experiment 2; ▲ pH.

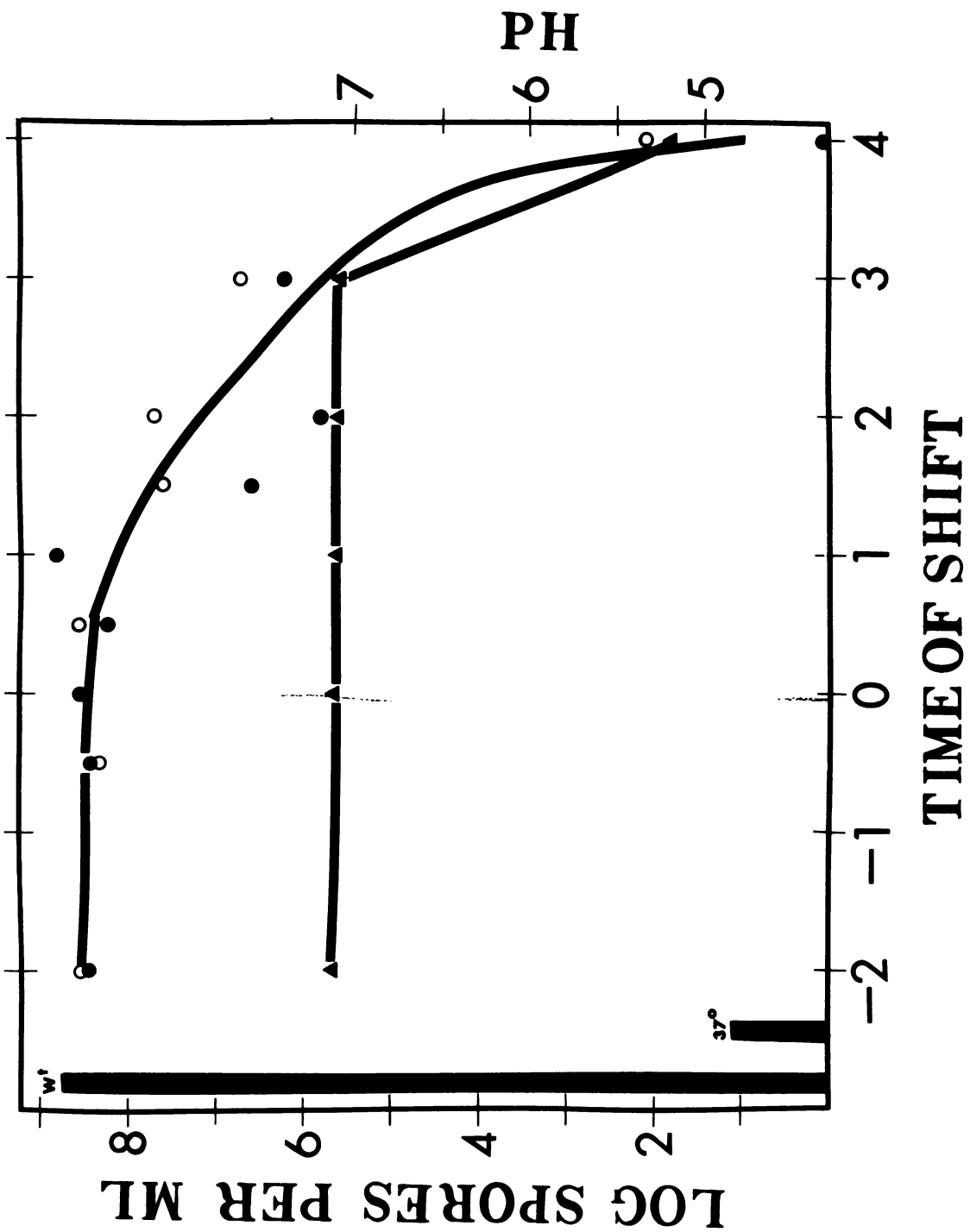

PH
7
6
5
TIME OF SHIFT
−2
−1
0
1
2
3
4
LOG SPORES PER ML
8
6
4
2
37°
W'

FIGURE 5. Sporulation of JS22-C cultures shifted-up from 26 C to 37 C at different times during the growth cycle. T_o designates the end of exponential growth. Heat-resistant spores were counted by plating spore suspensions at T_{30} after heating at 70 C for 30 min. The pH was measured at T_{30}. The bar designated W^t represents the spore yield in cultures of the parent strain which were shifted to 37 C at T_o. The bar designated 26 C represents the spore yields in cultures of JS22-C maintained at 26 C throughout the course of the experiment. Symbols: ● heat-resistant spores, experiment 1; O heat-resistant spores, experiment 2; ▲ pH.

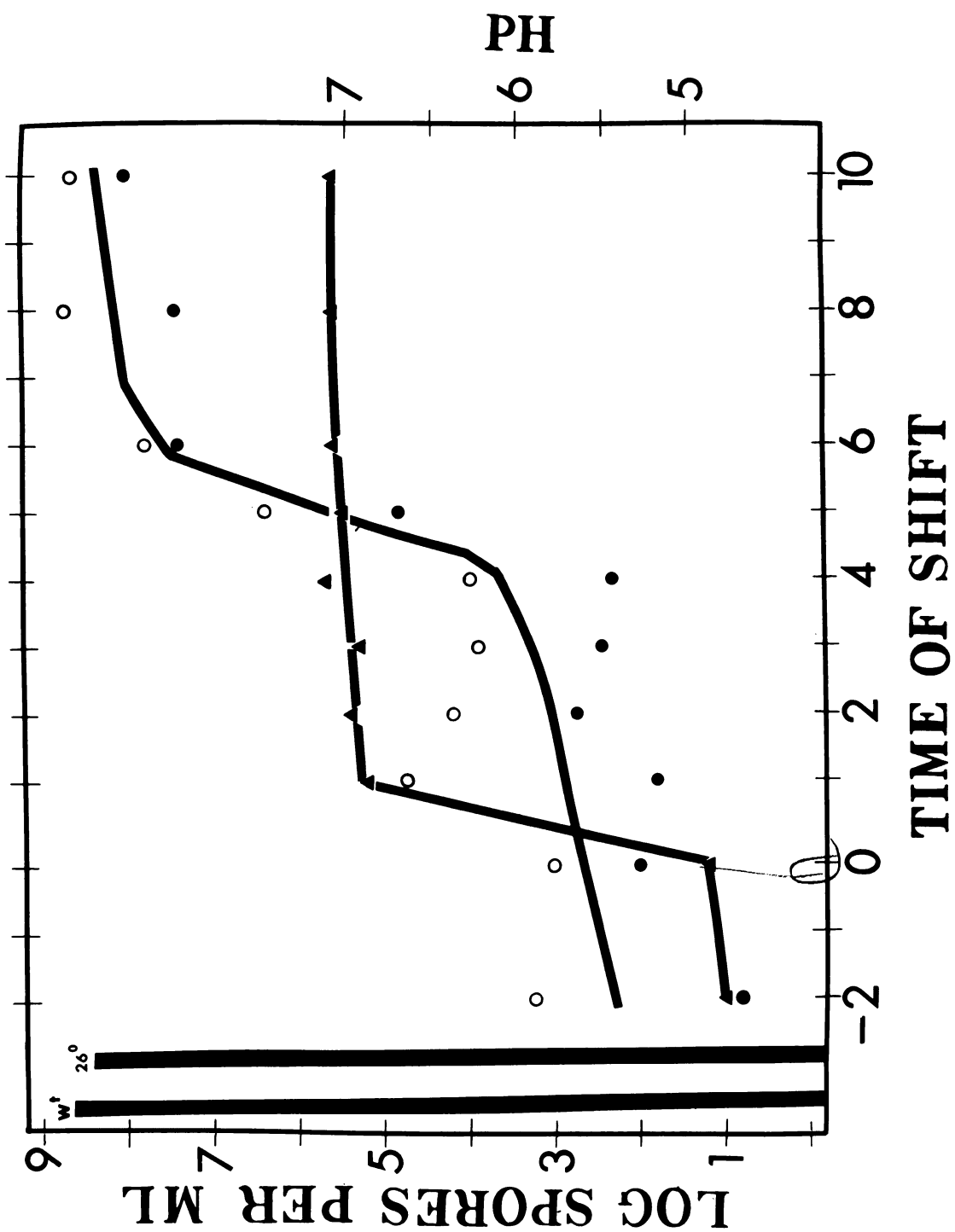
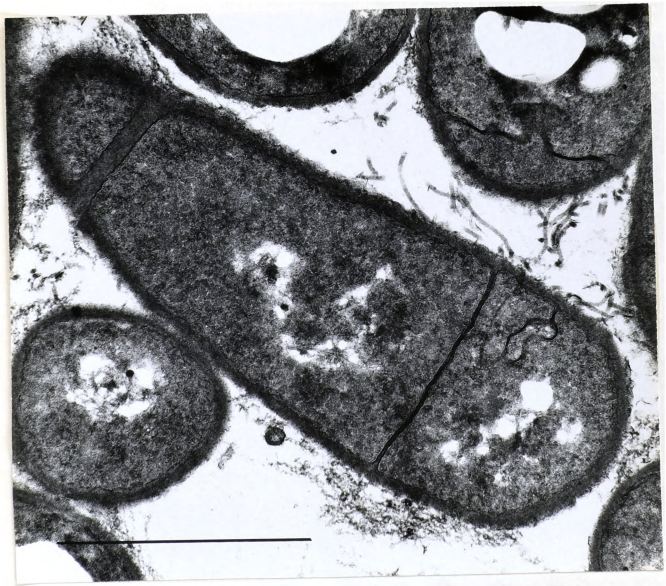

PH
7
6
5
10
8
6
4
2
0
−2
TIME OF SHIFT
LOG SPORES PER ML
9
7
5
3
1
w'
26°

FIGURE 6. Electron micrograph of a longitudinal section of one cell and part of a longitudinal section of a second cell from a sample of JS22-C removed at T_6 from a culture growing at 26 C. The bar in the lower left-hand corner represents one micron.



APPENDIX

APPENDIX

SOME FURTHER CHARACTERISTICS OF THE LP-22 - *BACILLUS CEREUS* T PHAGE-HOST RELATIONSHIP

(ABSTRACT)

The high frequency of induction of bacteriophage LP-22 by cells of temperature-sensitive sporulation mutant JS22 is accompanied by a high frequency of spontaneous curing in the mutant strain. This curing is not due to the occurrence of the phage genome as a non-integrating plasmid. Although the phage is induced in the mutant at high frequencies, it appears to be uninducible in the wild-type. Treatments with ultraviolet light, mitomycin C, and cis dichlorodiammineplatinum (II) and growth at elevated temperatures all fail to cause phage induction in the wild-type. Although 99% of the phage induced by mutant JS22 do not appear in the culture fluid until after the end of exponential growth, the phage does not appear to require sporulation specific RNA polymerase or protein synthesizing machinery for replication. The appearance of the phage at that time may be due to the fact that conditions for induction are less favorable during exponential growth. LP-22 does not appear to be capable of mediating generalized transduction.

LP-22, a bacteriophage of unusual morphology, was discovered in lysates of temperature sensitive (ts) mutants of Bacillus cereus T which were blocked in an early function of sporulation and which had concurrently become sensitive to lysis by the phage. Preliminary studies suggested a relationship between the inability of the mutants to sporulate and the increased sensitivity of the mutants to lysis by the phage. The mutants produced titers of phage as high as 3×10^9 plaque forming units (pfu)/ml; while the parent strain produced titers of 10^4 pfu/ml or less. Single step revertants of one of the mutants, JS 22, produced titers approximately the same as those produced by the parent strain. Over 99% of the phage released by the mutants did not appear until after the end of exponential growth. Many of the phage particles released by the mutants were morphological variants having tails of different lengths with unusual plate-like structures placed at irregular intervals along the tails and perpendicular to the length of the tails (8).

A better understanding of factors which influence the stability of virus-host relationships could be achieved through knowledge of the reasons for the disruption of the normally stable LP-22-B. cereus T phage-host relationship by mutation to asporogeny. This investigation was therefore undertaken to gain further

information on the nature of the LP-22-*B. cereus* phage-host relationship in both wild-type and mutant strains and to determine whether LP-22 could be used in genetic studies as mediator of generalized transduction.

MATERIALS AND METHODS

Bacterial Strains and Phage. The bacterial strains used in this study were a tryptophan auxotroph of *Bacillus cereus* T and two *ts* sporulation mutants, JS22 and JS29, which were derived from the try-auxotroph. The auxotrophs used in the transduction experiments, JS3 *pur* $^{-}$*Sm*^{r}, JS11 *tyr* $^{-}$*Sm*^{r}, and JS32 *arg* $^{-}$*Sm*^{r}, were all derived from wild-type *B. cereus* T. The procedures for cultivation and maintenance of these strains and for the propagation and assay of the phage (LP-22) were described in an earlier publication (8).

Screening for Clear-Plaque Mutants. In attempts to find spontaneous clear-plaque mutants, 10^{9} to 2.5 X 10^{9} pfu of LP-22 were added to 3.0 ml of soft nutrient agar (0.5% agar) which had been seeded with *B. cereus* try^{-}; the seeded agar was layered over nutrient agar, and the plates were incubated at 37 C. After 12 to 15h incubation the plates were examined for clear plaques. Mutagenesis by nitrous acid was accomplished by mixing 2.0 ml of LP-22 (1.5 x 10^{10} pfu/ml) with 1.0 ml 60mM sodium nitrate and 1.0 ml 1.0M sodium acetate buffer

(pH 4.2) and allowing the mixture to stand at room temperature. At 30-minute intervals samples were removed, diluted 1:10 in nutrient broth and added to soft agar seeded with B. cereus try$^-$. The last sample was removed at 3h. Hydroxylamine treatments consisted of mixing 0.5 ml LP-22 (1.5 X 10^{10} pfu/ml) with 1.0 ml 4M hydroxylamine, 1.0 ml 1.5M NaOH, and 2.5 ml 0.M $NaHPO_4$ buffer (pH 6.0) and incubating the mixture at 37 C. In the period 6h to 10h after incubation, samples were removed at 2h intervals, diluted 1:10 in nutrient broth and added to soft agar seeded with B. cereus try$^-$.

Detecting cured strains. Individual colonies of mutant JS22 and of the parent strain were tested for their capacity to produce LP-22 by three different methods. The first method consisted of replicating plates containing colonies derived from single spores unto plates which had been overlayed with soft nutrient agar (0.5% agar) seeded with JS22-C, incubating at 37 C and looking for lysis of the lawns. The second method consisted of overlaying the colonies with soft nutrient agar which had been seeded with JS22-C, incubating at 37 C and looking for zones of lysis around the colonies. The third, and most effective method, consisted of inoculating 5.0 ml nutrient broth with cells from single isolated colonies, growing the cultures overnight at

37 C and testing the supernatant fluids for phage by spotting 0.2 ml onto plates overlayed with soft agar which had been seeded with JS22-C.

Cesium chloride-ethidium bromide density gradients. Twenty ml of modified G medium (1) supplemented with casein hydrolysate (0.2%) in place of yeast extract, deoxyadenosine (250 g/ml), and ^{3}H thymidine (0.1 m Ci, New England Nuclear) were inoculated with 2 X 10^{8} spores of B. cereus try^{-} or JS22 and were grown at 37 C until early stationary phase. The cells were pelleted by spinning at 10,000 rpm for 10 min (4 C) and were washed with cold TES buffer [0.02m tris (hydroxymethyl) aminomethane (Tris), 0.01 MCaCl, 0.005 M ethylenedromine-tetraacetic acid (EDTA), pH 7.5]. Lysates were prepared by the method of Lovett (4), with one modification: immediately after pronase treatment the cells were frozen and thawed six times to achieve complete lysis. The final volume of the lysate was adjusted to 5.0 ml with TES buffer, and 6.9 grams of CsCl (General Biochemicals) was added. A 7.0 ml amount of the resulting solution was mixed with 3.0 ml ethidium bromide (EB; Sigma, 4 mg/ml in 0.1 M phosphate buffer pH 7.0) and distributed equally between two polyallomer tubes. The tubes were topped with mineral oil, and spun for 42 h at 36,000 rpm in a Ti 50 fixed angle rotor at 15 C in a Beckman model L3-50 preperative ultracentrifuge (4). Seven drop fractions (approximately 0.15 ml) were

collected through holes pierced in the bottoms of the tubes, and 10 µl portions from each were spotted on squares (1.0 cm) of Whatman no. 1 filter paper. The squares were dried, washed in cold 10% trichloraacetic acid (TCA), rinsed in 95% ethanol, and dried. After drying, the squares were placed in scintillation vial containing 5.0 ml toluene scintillation fluid [toluene containing 0.4% 2, 5 diphenyloxazole (PPO) and 0.005% 1, 4 -di- 2 - (5 phenyloxozolyl) - benzine (dimethyl POPOP)] and counted on a Packard Tricarb liquid scintillation counter.

Induction of LP-22. Several procedures were used in attempts to induce LP-22 from wild-type B. cereus. In the first procedure, approximately 10^8 cells were suspended in 6.0 ml sterile saline in a glass petri dish and were exposed to ultraviolet light (G.E. 30 watt germicidal lamp) at a distance of 45 cm for 30 sec. The cells were agitated constantly during the exposure. The surviving cells (approximately 37% of the initial number) were used to inoculate 500 ml flasks containing 50 ml nutrient broth. These cultures were grown at 37 C for periods ranging from 2h to 5h and the number of pfu of LP-22 in the supernatant fluids determined by plaque assay on lawns of JS22-C (8). Both exponentially growing cells and sporulating cells were subjected to this treatment.

In the second procedure, 5.0 ml subcultures were removed from a 100 ml culture of wild-type B. cereus which was growing in modified G medium at 37 C and were treated with 2 mg/ml mitomycin C for 10 min. The cells were spun down at 10,000 rpm for 10 min., washed once with 0.1 M phosphate buffer (pH 7.0) and resuspended in modified G medium with no glucose. After 5h incubation at 37 C the cultures were assayed for the number of phage in the supernatant fluids. The subcultures were taken 2h before the end of exponential growth; at the end of exponential growth, and 1h, 2h, and 3h after the end of exponential growth.

In the third method, cultures of wild-type B. cereus growing in nutrient broth at 37 C were treated with cis-dichlorodiammineplatinum (II) at concentrations ranging from 0.1 μmolar for a period of 2.5h (7); and supernatant fluids were assayed for phage. The final method consisted of growing wild-type B. cereus in nutrient broth at 45 C and assaying supernatant fluids of exponentially growing cultures and of stationary cultures for phage.

Production of phage during exponential growth. Early exponential cultures of JS22-C and JS29 were infected with LP-22 at a multiplicity of infection of 1.0. Growth of infected cultures and uninfected control cultures was followed by measuring OD_{620} at 30 min.

intervals. The number of phage in the culture supernatant fluids were assayed on lawns of JS22-C.

Isolation of auxotrophs. Nutrient broth cultures of wild-type B. cereus were harvested during mid-exponential growth by spinning at 10,000 rpm for 15 min. The cells were washed with an equal volume of tris-maleic acid buffer (pH 6.0), resuspended in 50.0 ml of the same buffer containing 100 µg of N-methyl-N-nitro-N-nitrosoguanidine (NTG, Aldrich Chemical Co.), and incubated with shaking at 30 C and 200 rpm for 30 min. Normally 80 to 90% of the cells were killed during this treatment. The cells were washed with 50.0 ml double strength nutrient broth, resuspended in 50.0 ml fresh double strength nutrient broth, and incubated on a reciprocal shaker at 30 C and 200 rpm for 3h, to allow for segregation of the mutant genomes and dilution of the wild-type enzymes. The cells were again spun down at 10,000 rpm for 15 min., washed with minimal medium (5), resuspended in minimal medium containing 200 µg D-cycloserine/ml (Nutritional Biochemicals) and incubated on the shaker for 5h at 30 C. During this treatment approximately 99% of the cells were killed. The cells were again spun down, washed with double strength nutrient broth, to remove the D-cycloserine, and grown in double strength nutrient broth for 6h at 30 C and 200 rpm.

Samples were removed from the enriched culture, diluted to yield about 100 colonies per plate, and spread on nutrient agar plates. After the plates had been incubated for 12h at 30 C, replicate plates were made on minimal agar. Colonies which failed to grow on the minimal medium after 8h incubation at 30 C were restreaked on minimal medium and incubated overnight. All strains which failed to grow were screened for specific growth requirements on plates of minimal agar supplemented with pools of various amino acids, vitamins, and nitrogen bases. Streptomycin resistant strains were derived from each stable auxotroph for use in transduction experiments.

Transduction. The phage used in the transduction experiments were obtained from infected cultures of JS29. Optimal numbers of pfu/ml were obtained from nutrient broth cultures infected during early exponential growth at a multiplicity of infection of 0.1. The phage suspensions were passed through a 0.45 filter (Gelman) before their use in the experiments. The procedures used in the transduction experiments were the same as those of Yelton and Thorne (10) except each set of plates was done in triplicate rather than in duplicate, and the experiments were repeated with cells which had reached the end of exponential growth. Multiplicities of infection varied from 1.0 to 3.0.

RESULTS

Clear-plaque mutants. Although large numbers of phage have been treated with nitrous acid and hydroxylamine, no clear plaque mutants have been isolated. The lack of clear-plaque mutants makes it impossible to study phage replication in the wild-type at this time.

High frequency of spontaneous curing. The first indications that mutant JS22 became cured of the LP-22 genome at a high frequency were the observations that serial subculturing of single colonies produced cured strains (8) and from data concerning the stability of phage in heated and unheated spores (Table 1). Colonies derived either from heat-shocked spores or from unheated spores were overlayed with soft agar seeded with JS22-C, and the plates were incubated at 37 C for 12h.. The colonies which produced phage caused a localized clearing of the indicator strain. The number of colonies producing detectable titers of phage increased with the age of the colonies. After 24h the entire lawns underwent lysis; so it was not possible to determine the percentage of colonies capable of producing phage after longer periods

of incubation. Only a little over one-third of the colonies derived from single spores produced detectable phage. This was true of colonies derived from heat-shocked spores and from unheated spores (Table 1). These data indicated that nearly two-thirds of the spores had either become cured or were not induced until they were very old.

Since the agar overlay method did not appear to be a highly sensitive method for measuring the capacity of an individual colony to produce phage, the experiment was repeated using a more sensitive technique. Individual colonies, which had been derived from spores, were used to inoculate tubes containing 5.0 ml nutrient broth. The cultures were incubated for 24h, spun down at 10,000 rpm for 10 min. and 0.2 ml samples from each supernatant fluid were spotted on sensitive lawns. After 12 h incubation at 37 C, the plates were examined for lysis. Only 11 of 20 cultures produced phage. The colonies which did not produce phage had become sensitive to plaque formation by the phage; therefore they appeared to be cured.

Attempts to repeat this experiment with the parent strain were not successful. Cultures of the parent strain sometimes produced as few as 5 to 10 pfu/ml, and these were not always detected during the first screening. In order to obtain higher phage yields

from the parent strain so that cured strains could be readily observed, a means of inducing the phage was sought. Attempts to induce the phage by treatment with ultraviolet light, mitomycin C, and cis-dichlorodiammineplatinum (II) all failed both in exponentially growing cells and in sporulating cells. Attempts to induce the phage by raising the temperature of incubation to 45 C also failed.

Cesium chloride - ethidium bromide density gradient centifugation. The higher frequency at which cured strains of JS22 were found would not be expected to occur in cells carrying an integrated prophage; therefore the possibility that LP-22 was carried as a nonintegrating plasmid was investigated. Cleared lysates obtained from wild-type B. cereus, which had been labeled with ^{3}H thymidine, were layered on a cesium chloride-ethidium bromide solution and subjected to centrifugation until equilibrium was reached.

Figures 1 and 2 show that there was only one peak of radioactivity in the gradients. If plasmid DNA had been present a second, smaller, peak composed of covalently-closed circular DNA should have been present in the denser fractions nearer to the bottom of the tube (6). A control experiment was performed using E. coli JC 411 (col. E.), and the results indicated that plasmid DNA was present (data not shown).

Although the LP-22 genome did not appear to occur as a nonintegrating plasmid in the wild-type cells, it was possible that it was unable to integrate properly, and did occur as a plasmid in JS22. To determine whether JS22 carried the phage as a plasmid, cleared lysates of that strain were subjected to the same procedure used for the wild-type. The results (Figure 2) indicated that JS22 did not carry plasmid DNA.

Infection of exponentially growing cells. The observed release of 99% of the total pfu of LP-22 by mutant JS22 after the end of exponential growth (8) could have been due to infection of the cured cells by the few phage which were released during exponential growth combined with an unusually long latent period. Alternatively it could have been due to the fact that conditions during exponential growth were less favorable either for phage induction or for phage replication.

It was of particular interest to determine whether exponentially growing cells were able to replicate the phage since failure to do so could indicate a requirement by the phage for a sporulation-specific RNA polymerase or sporulation-specific protein synthesizing machinery. The ideal way to determine whether the phage could be replicated in vegetative cells would be to infect the wild-type cells with a clear-plaque mutant and to look for massive lysis accompanied by a large increase in

the number phage in the supernatant fluid. Since no clear-plaque mutants have been found, the experiment was performed by infecting JS29, a mutant that has lost its superinfection immunity and forms clear plaques (8). A nutrient broth culture of JS29 was divided into two portions; one was infected during the early stages of exponential growth at a multiplicity of infection of 1.0; the other was not infected. The growth of both cultures was followed by measuring the OD_{620} on a B. and L. Spectronic 20 spectrophotometer. The final number of pfu/ml was measured at 2.5h by standard plaque assay methods. Figure 3 shows that lysis did occur during exponential growth. The phage titers 2.5h after infection had risen from an initial 10^7 pfu/ml to 9×10^9 pfu/ml indicating that the drop in OD_{620} was not due to lysis from without but to the production of bursts of phage by the infected cells. When the same experiment was performed with JS22-C very little lysis was observed, but phage titers did increase from an initial 3.5×10^6 pfu/ml by the end of exponential growth. The number of phage present in the supernatant fluid at the end of exponential growth was approximately 35% of the total number found 5.5h later. These data indicate that the phage could be replicated and assembled by vegetative cells. The data from the experiment in which JS22-C cells were infected imply that most of the

phage enter into a stable lysogenic or pseudolysogenic relationship with that strain, rather than producing a burst.

Transduction. It was of great interest to determine whether LP-22 was capable of mediating generalized transduction; since only two phage have been shown to mediate generalized transduction in B. cereus, and only one of these is effective with B. cereus T (9, 10, 11).

Preliminary experiments to determine whether LP-22 could mediate generalized transduction were performed using three auxotrophs: JS3 ($pur^{-}Sm^{r}$), JS11 ($try^{-}Sm^{r}$), and JS32 ($arg^{-}Sm^{r}$) as recipients. Although a few spontaneous revertants of strains JS3 and JS32 were observed, no transductants were observed in any experiments.

DISCUSSION

The loss in the ability of 45% of the strains derived from single spores of mutant JS22 to produce plaque-forming particles of phage LP-22, accompanied by the acquisition of sensitivity of these strains to plaque formation by LP-22, strongly suggests that these strains have been "cured" of the phage genome. This high frequency of curing is not due to the occurrence of the LP-22 genome as nonintegrating plasmid in the host cells. This high frequency of curing has not been seen in the parent strain, and the parent strain appears to carry the phage as a stable lysogen (8). Therefore, it is probable that the genetic lesion that occurred in JS22 has altered that mutant in such a way that it is no longer able to carry the phage as a lysogen but carries it as a pseudolysogen. The pseudolysogenic relationship of phage SP-10 and Bacillus subtilis (3) shows some similarity to the relationship between LP-22 and JS22. Cultures of B. subtilis W-23-S^r produce high titers of SP-10 and, simultaneously, a high frequency of cured cells. The cured cells are believed to result

from a lack of coordination in the replication of the phage genome and the bacterial genome which causes the phage genome to be unavailable for distribution to both daughter cells at division time.

The observed ability of LP-22 to replicate in exponentially growing cells of JS29, a mutant which has lost superinfection immunity, indicates that the phage probably does not require a sporulation-specific ribonucleic acid polymerase or sporulation-specific protein synthesis in order to replicate. It is unlikely that JS29 vegetative cells possess a sporulation-specific RNA polymerase or a modified protein synthesizing system since they grow normally at the restrictive temperature and are blocked at stage 0 of sporulation. It would be desirable to verify this result by infecting the parent strain with a clear-plaque mutant of LP-22, but no clear-plaque mutants have been isolated at this time. Determination of the latent period and burst size also depend on the isolation of a clear-plaque mutant. Single step growth studies using JS29 as the host have not been successful due to a high "background" caused by the spontaneous induction of phage by the cells.

The inability of LP-22 to transduce three auxotrophic mutants which have lesions in three unrelated biosynthetic pathways indicates that it is unlikely that LP-22 is capable of mediating generalized transduction in Bacillus cereus T.

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TABLE 1. Heat stability of phage carried in spores of JS22

Hours after overlaying	Colonies derived from unheated spores			Colonies derived from heat-shocked spores[a]		
	total colonies	colonies producing phage	percent producing phage	total colonies	colonies producing phage	percent producing phage
12	488	118	24	612	131	21
24	464	165	36	591	207	35

[a]Heat-shocking was carried out at 70 C for 30 min.

FIGURE 1. CsCl-ethidium bromide gradient centrifugation of DNA extracted from Bacillus cereus try⁻. DNA, labeled with ^{3}H-thymidine, was extracted as described in Materials and Methods. The symbol ≈ signifies a change in the scale of CPM from the left-hand side to that on the right in counts of 10^{3} cpm.

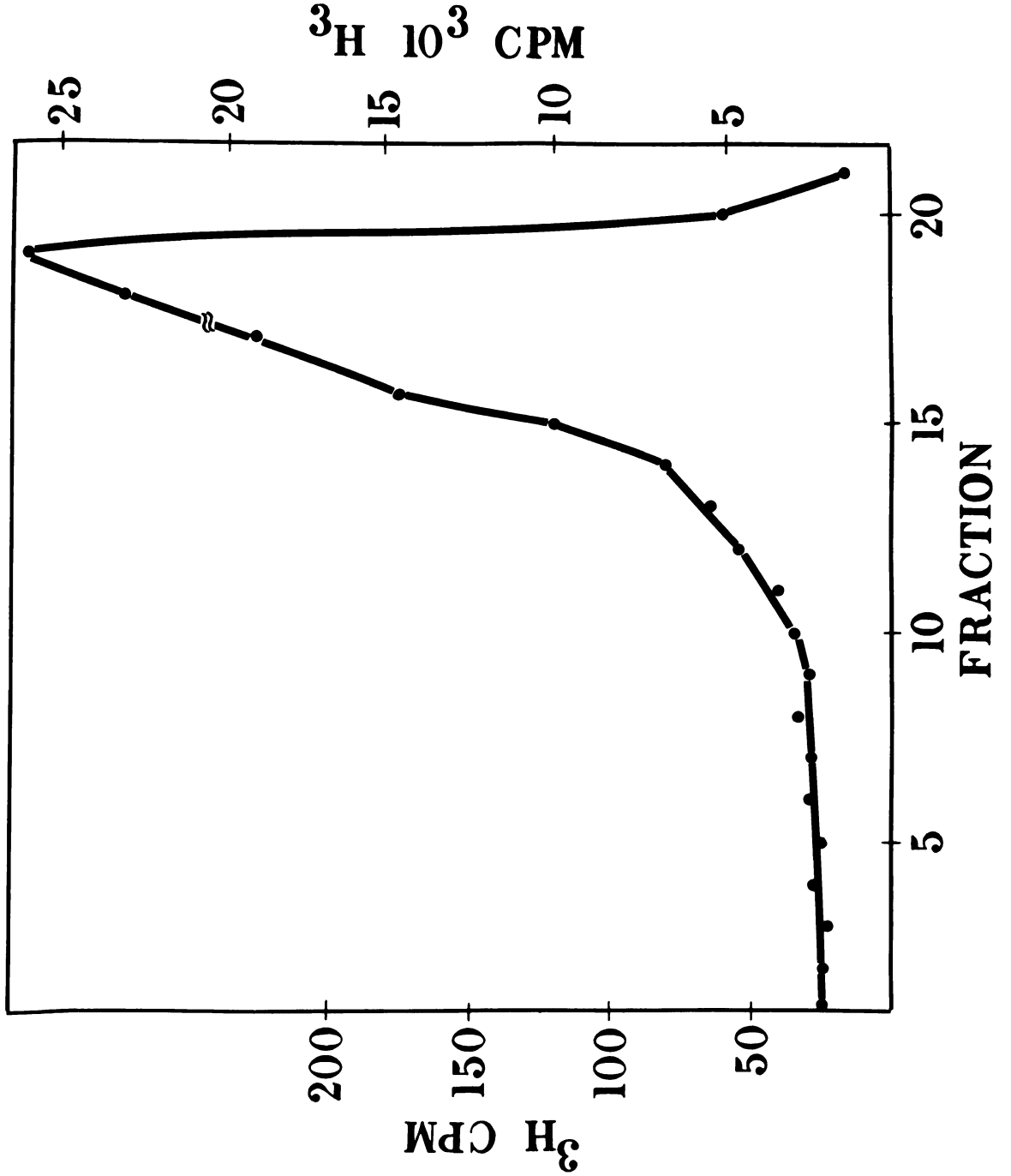

^{3}H 10^3 CPM
25
20
15
10
5
^{3}H CPM
200
150
100
50
5
10
15
20
FRACTION

FIGURE 2. CsCl-ethidium bromide gradient centrifugation of DNA extracted from Bacillus cereus JS22. DNA, labeled with ^{3}H-thymidine, was extracted as described in Materials and Methods. The symbol $\approx$ signifies a change in the scale of CPM from the one on the left-hand side to that on the right in counts of 10^{2} cpm.

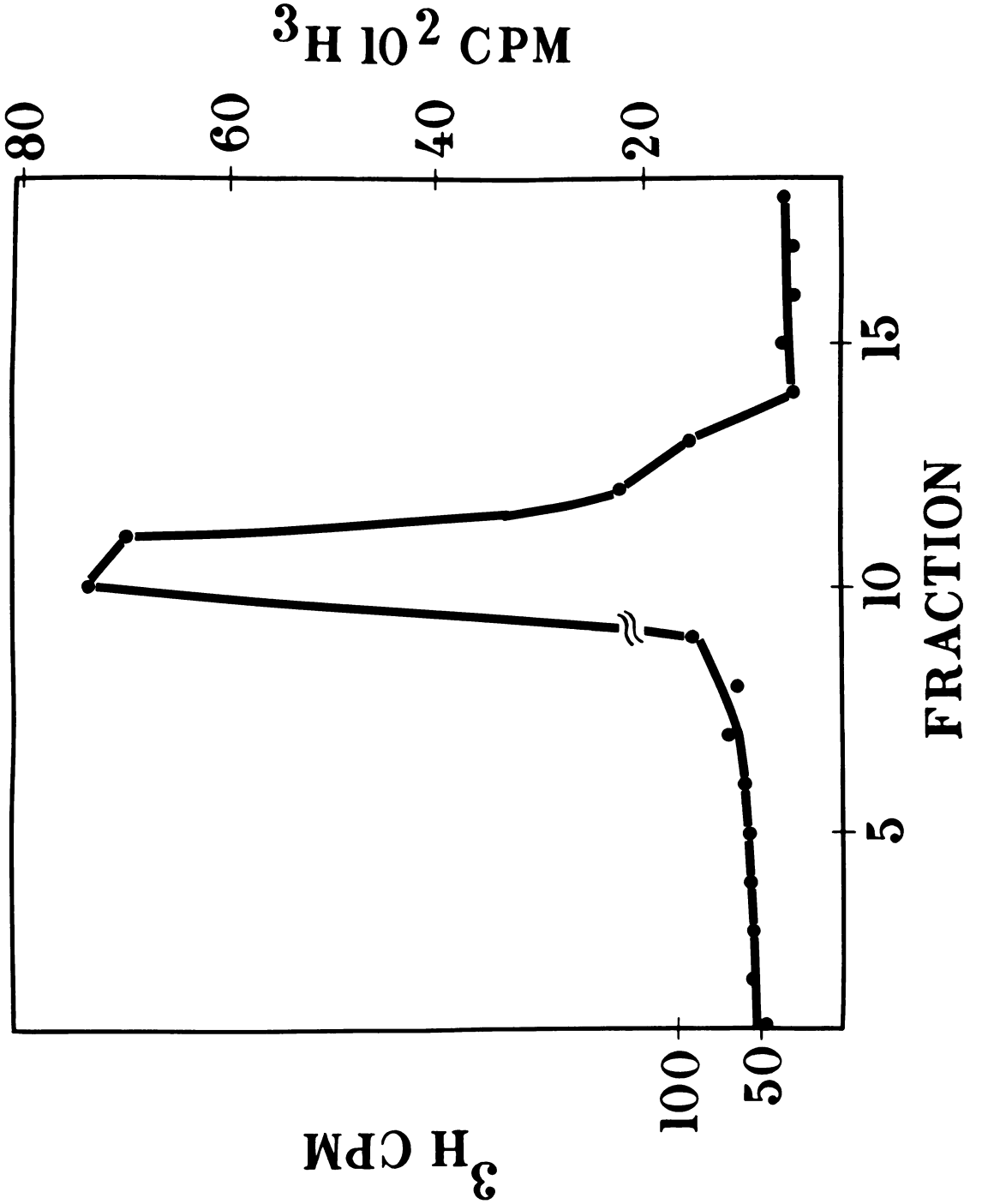

^{3}H 10^2 CPM
80
60
40
20
15
10
5
FRACTION
100
50
^{3}H CPM

FIGURE 3. Growth and lysis of a broth culture of Bacillus cereus JS29 infected with LP-22 at a multiplicity of infection of 1.0. Symbols: ●, OD_{620} of an uninfected control culture; O, OD_{620} of the infected culture.

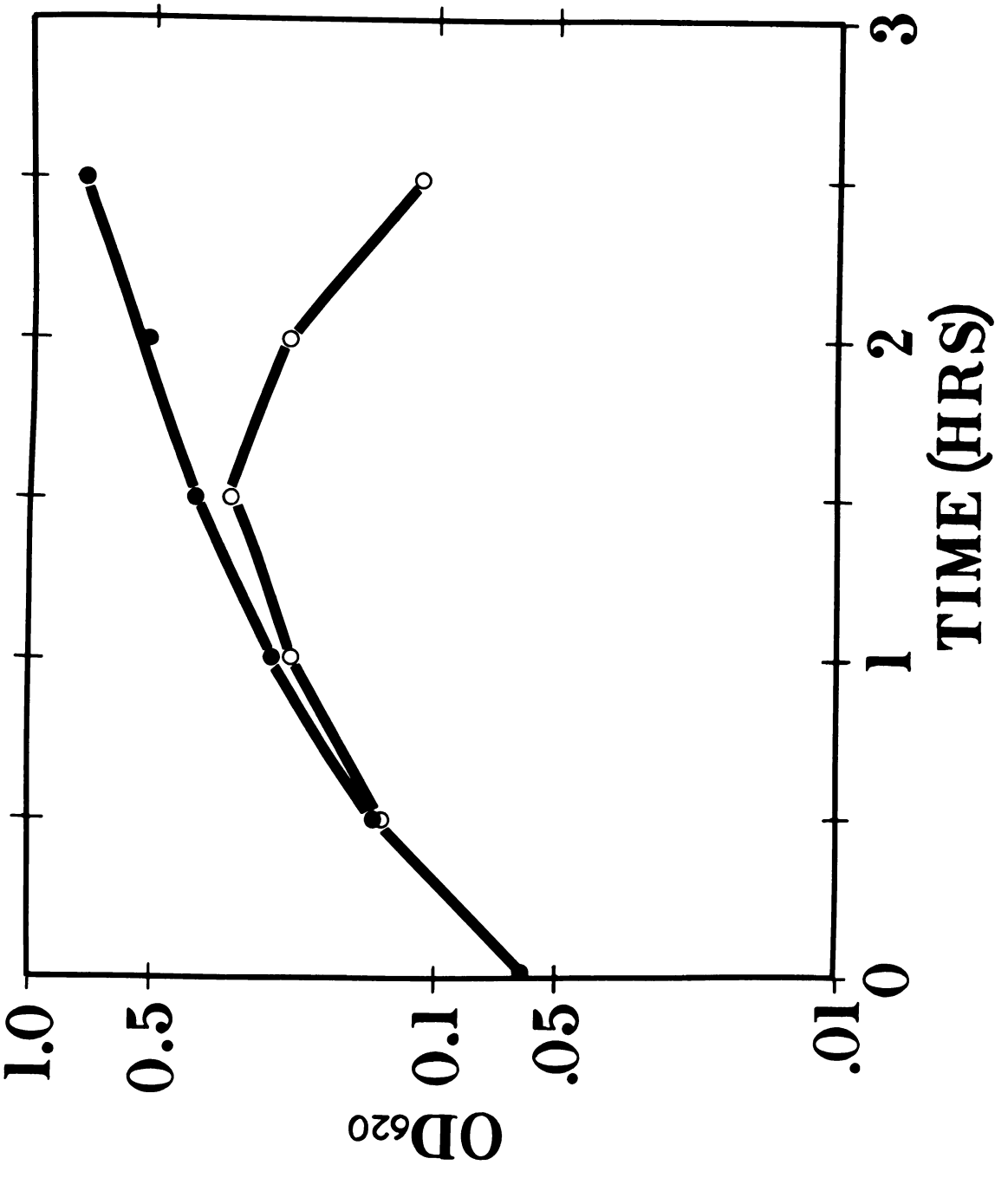
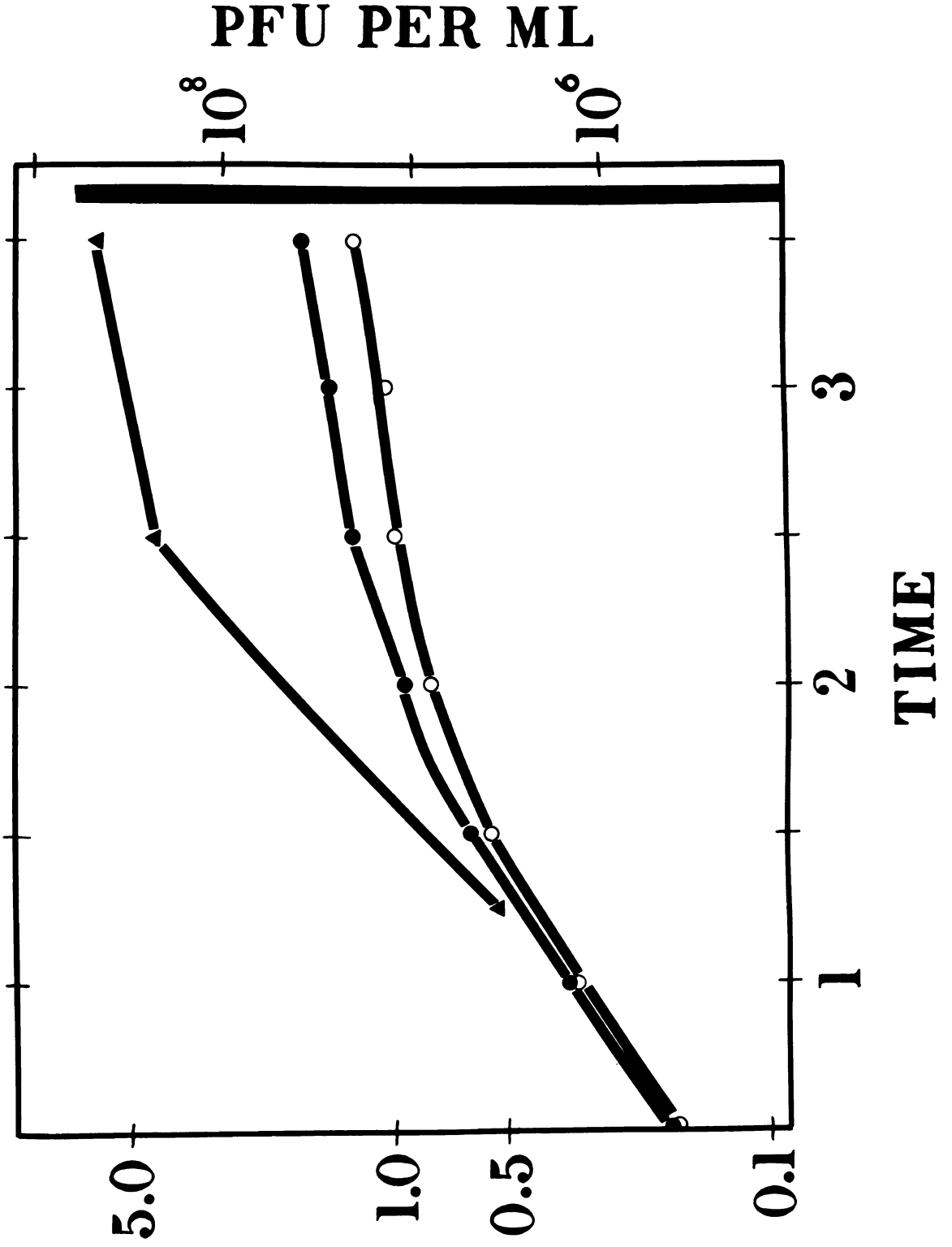

1.0
0.5
0.1
.05
.01
OD620
0
1
2
3
TIME (HRS)

FIGURE 4. Growth and lysis of a broth culture of Bacillus cereus JS22-c infected with LP-22 at a multiplicity of infection of 1.0. Symbols: ●, OD_{620} of an uninfected control culture; O, OD_{620} of the infected culture; ▲, pfu/ml.


PFU PER ML
10^8
10^6
5.0
1.0
0.5
0.1
1
2
3
TIME