CHARACTERIZATION OF A COMBINED DNA INITIATION
AND CELL DIVISION MUTANT OF BACILLUS SUBTILIS

by

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ABSTRACT

Bacillus subtilis 168-134 (ts), a DNA initiation mutant, was transferred to the minicell producing strain, CU403 Div IV-B1, to study the relationship of DNA synthesis to cell division. Markers in the combined mutant were verified by transduction. DNA replication kinetics, genome location by autoradiography, and clonal analysis of cell division patterns during spore outgrowths were investigated. Growth at the restrictive temperature results in an impressive reduction of the per cent cell length covered by DNA grain clusters (60% at 30 C compared to 8% after 2 h at 45 C). The probability of a minicell producing division appears essentially the same at 30 C and during the initial 2-3 h growth at 45 C at which time numerous clones begin to lyse; most likely residual division at 45 C is attributable to processes initiated at 30 C rather than an authentic expression of the DNA initiation gene's action at 45 C. Of interest is that CU403 Div IV-V1, 134 (ts) divides about 25% as frequently relative to growth as do wild type CU403 clones. This is approximately 15% greater division suppression than previously found in the CU403 Div IV-B1 mutant strain. Additional studies are in progress which hopefully will permit a true exposure of the interrelationship between the DNA initiation and the minicell producing genes.

INTRODUCTION

Cell division remains a poorly understood phenomenon despite the scientific efforts of innumerable investigators. The processes of DNA synthesis, protein synthesis, and cell division are enigmatically enmeshed and difficult to untwine; therefore, it is imperative to consider them concomitantly.

What is involved in division simply is the increase in cell mass, replication of DNA, segregation of nuclear material, partitioning of the parent cell into two daughter cells by septum formation and cleavage of these cells (82, 97). All layers of the envelope are implicated. In gram positive rod-shaped organisms, division seems to be initiated morphologically by a growth inwards at right angles to the length of the cell of a ring of wall material (32, 69). L-forms, protoplasts, and mycoplasma which lack the rigid cell wall comprised of peptidoglycan and teichoic acid are able to divide. Thus the absence of a cell wall does not preclude division (14, 18).

Correlation of Division With Cell Mass

Division is correlated with the attainment of a cellular "unit of mass" (16). Initiation of a round of DNA replication always takes place when the cell mass per chromosome origin reaches a particular critical value except in mutants where this might vary. Mass at initiation is an integral multiple of a particular mass. More DNA is formed with increasing growth rates because new rounds of replication

begin every τ minutes where τ is the generation time (31, 82). This constancy provides an explanation for enlargement of the size of cells with an enhancement in the rate of growth due to the increased number of chromosome origins or termini per cell (16, 55, 82).

Constancy of Cell Division and DNA Replication

It has been found in Escherichia coli B/r growing at 37 C chromosome replication takes about 40 min followed by cell division within 20-25 min. During the division period, the time between termination of chromosome replication and the commencement of cell division, division proteins (produced concurrently with DNA synthesis) are assembled or activated (30, 31, 55).

Regardless of the time required for the population to double, cell division and DNA replication appear constant pertaining to the interim needed for the processes to occur as well as to their inter-relationship (chromosome replication equals 2 x division period) (72) except in exceedingly slow growing cells with doubling times greater than 60 minutes and in certain DNA mutants (20, 73).

The DNA content per unit of cell mass remaining steadfast over a wide range of growth rates can be attributable to the fact that the rate of travel of the DNA replicase is essentially the same but the amount of DNA synthesis is governed by the number of chromosome copies being simultaneously replicated (30, 68, 77). However, it has been shown by Pritchard and Zaritsky (76) that the replicase travel time can vary in a thymine-less mutant when the cells are grown in different

thymine concentrations but most models of the cell cycle consider the replicase travel time constant.

Dependency of Cell Division On DNA Synthesis

Investigators (11, 12, 17, 41, 82) believe that cell division depends on DNA replication and genome segregation, since events leading to division are regulated precisely. This results in newborn cells that are quite uniform in size for a specific growth rate and anucleate cells are rare. Any cell which develops a control mechanism for the segregation of equal portions of genetic material among progeny is at an advantage with respect to survival (41).

Frequently, DNA synthesis inhibition prevents cell division (11, 12, 13, 17), whereas cell division curtailment does not necessarily affect DNA replication (45, 67, 79, 88); this reliance of cell division on DNA synthesis insures that division only occurs in cells which have replicated and segregated their DNA as proposed by Jacob, Ryter, and Cuzin (41).

Cell division at different growth rates is believed to be determined by the rate of DNA synthesis which is governed by the frequency with which rounds of replication are started, initiation being more often at rapid growth rates (37, 75, 82).

Role of Protein Synthesis

Protein synthesis is imperative for the initiation of these rounds of DNA replication although rounds already inaugurated proceed to completion when protein synthesis is blocked (18, 31, 49, 50, 55, 101). The dependence of DNA synthesis initiation on continuing protein

synthesis permits a way of altering the rate of DNA replication with changing growth rate.

Mechanisms Regulating DNA Synthesis Initiation

Two mechanisms have been proposed for regulating DNA synthesis. One is by a positive means whereby an hypothetical initiator protein is continuously formed as a constant fraction of the total protein. Initiation occurs when the initiator protein reaches a certain level (30).

A negative control mechanism has been offered by Pritchard, Barth, and Collins (75), who declare that a positive initiator protein is present under all conditions at a sufficient level to allow initiation. Whether or not inception takes place is determined by the quantity of inhibitor (H) which interacts allosterically with the initiator protein/s. Immediately following initiation, the gene specifying this inhibitor doubles and only during replication is the gene transcribed and the inhibitor formed. Thus, the level of the inhibitor rises and obviates further initiations. With the growth of the cell, the amount of the inhibitor falls with the initiations occurring when the concentration has been halved. The quantum of inhibitor oscillates between H at initiation and 2H afterwards.

Not only is protein synthesis essential for commencement of DNA replication but division proteins are produced in a separate pathway concurrently with DNA synthesis during the first 40 min subsequent to initiation in E. coli at 37 C (18, 42, 73).

Termination of DNA Synthesis

Also it has been found (11, 12, 24, 28, 31, 42, 57) that the termination of DNA replication is necessary for cell division and this means the rate of division will be governed by the time of replication termination and hence indirectly by replication initiation. Apparently with the completion of DNA replication, genes are transcribed for termination proteins which are required if cell division is to follow in 20-25 min. However, during this interval between cessation of DNA replication and the impending division, cell division can proceed in the presence of protein and DNA inhibitors indicating that during this time the preformed proteins begin functioning on are merely assembled and septum formation completed.

Jones and Donachie (43) have further proposed that the termination protein/s is required to release the replicated DNA from its membrane site thereby permitting division to take place. At the time of investigation of DNA replication the chromosome would once again become attached to the membrane site thereby reinstating the division block.

Variations in the Coordination of DNA Replication, Protein Synthesis, and Cell Division

Although evidence exists for the coordination of the DNA replication period (C), protein synthesis, and the cell division interval (D), investigations reveal the timing of these processes can be varied (87), such that the time between initiation of DNA replication rounds can be changed without affecting the DNA replication period or the division span (48); the C period can be altered without affecting D (56, 107); the D interval can be changed without modifying C (42, 88); and both

can be altered but not the relationship between C and D ($C = 2D$) (73). Pierucci and Helmstetter (73) suggest that a common mechanism controls chromosome replication and the progression of the cell towards division after completion of a round of replication since at all temperatures and in all media $C = 2D$ prevails (90).

For reasons previously mentioned DNA synthesis and cell division appear correlated (11, 12, 13, 31, 35) but numerous exceptions have not been noted (21, 31, 33, 34, 37, 38, 84, 85, 90, 91). Therefore, by what alternate process is cell division able to occur in the absence of DNA synthesis? Consequently, it was my intention to examine further the DNA synthesis-cell division interrelationship.

Temperature Sensitive DNA Initiation Mutants

Temperature sensitive mutants defective in DNA synthesis have been isolated in E. coli by Fangman and Novick (24), Kohiyama (47), Inouye (39), Kuempel (49), and Hirota, Ryter, and Jacob (35); in B. subtilis by Mendelson (59, 60), Mendelson and Gross (64), Gross, Karamata, and Hempstead (28), Karamata and Gross (44), Bazill (5), and Bazill and Retief (6); and in S. typhimurium by Spratt and Rowbury (90).

A DNA mutant can be affected in any of three processes: precursor synthesis, initiation of replication, or chain elongation. DNA initiation mutants belong to group B in B. subtilis (28) and to groups A and C in E. coli (34).

Various criteria are cogent in establishing a DNA mutant as being one belonging to the initiator class. Initiation mutants complete

rounds of replication which are underway at the time of transfer to the restrictive temperature but are unable to instigate new rounds, which is the critical test of proof for a DNA initiation mutant.

Other means of substantiating credibility as to the authenticity of a DNA initiation mutant is that the amount of DNA synthesized (40% in single fork asynchronous culture) at the restrictive temperature is similar to that produced in a protein inhibited (CAM, puromycin) exponential ts⁺ culture which is believed to prevent initiation of new rounds of replication (55). Depending on growth rate, completion of replication without re-initiation would result in an increase of DNA between 38 and 84%. Additionally, lambda (λ) possessing its own initiator can generally propagate in a DNA initiation mutant (24). Also transforming DNA from a DNA initiation mutant that has been incubated at the restrictive temperature for 1 h exhibits the relative transforming efficiency for origin and terminus markers characteristic of completed chromosomes (23, 51, 92, 93, 98, 102, 103). Furthermore, insertion of a plasmid into the bacterial chromosome produces a structure consisting of two replicons in tandem. If the plasmid codes for its own initiation of DNA replication it can "rescue" a DNA initiation mutant at the restrictive temperature as a result of passive replication (27).

Effect of DNA Synthesis Inhibition On Cell Division. Ordinarily a DNA initiation mutant at the nonpermissive temperature permits division, resulting in "normal" cells each containing a nuclear body, for approximately the time required for chromosome replication and division

(C + D) after which the cells elongate forming filaments, with the DNA remaining in the medial region of the cell (31).

If DNA synthesis is blocked by thymine starvation or nalidixic acid, cells continue to grow but do not divide. However, immediately upon addition of thymine a new round of replication is instituted at every chromosome origin whenever cell mass per chromosome origin reaches a critical value and cell division recommences after a delay which increases as a function of the length of the preceding period of initiation of DNA synthesis (17).

Kogoma and Lark (46) also have observed that if E. coli cells are thymine-starved or treated with nalidixic acid for 50-60 min, they not only begin new rounds of replication as soon as DNA synthesis is permitted, but are able to instigate additional rounds at a reduced rate for many hours in the absence of protein synthesis. This phenomenon does not occur after exposure of dnaB mutants to high temperatures.

Cell Division In the Absence of DNA Replication. Exceptions have been noted where cells proceed to divide in the absence of DNA synthesis. In E. coli, divisions in the presence of DNA inhibitors have only been observed in mutant populations where an alternate mechanism most likely functions.

For instance, Salmonella typhimurium 11G, (90) a DNA initiation mutant, displays two division phases: 1) 60-75 min after transfer to the restrictive temperature the cells divide in the usual fashion with nuclear segregation producing "normal" cells and 2) following a short

lag and 80-90 min succeeding transfer a second phase is observed resulting in short filaments which bud off at both ends.

Inouye (37, 39) observed that in Escherichia coli MX74T2ts27 division, upon immediate curtailment of DNA synthesis, was inhibited at high temperature yet at low temperature in the absence of thymidine no division occurs. It is suggested an uncoupling occurs due to heat sensitivity of a factor which links cell division with DNA replication (38, 86).

However, in Bacillus subtilis wild type cells are able to divide in the absence of DNA synthesis (21) which results in anucleate cells when the DNA rate of synthesis becomes lower than the rate of cell growth.

Production of Minicells

Anucleate cells both in Escherichia coli and Bacillus subtilis not only can result from DNA synthesis inhibition, but can arise as a consequence of loss of control of proper site location in cells which synthesize DNA in the customary manner.

The division process that yields minicells seems to be normal in many ways, for it involves a simultaneous invagination of wall and membrane and proceeds at a rate comparable with that observed for "normal" cell divisions. However, of importance is the fact that the nuclear region of the cell does not seem to be directly involved as DNA is not distributed to both sides of the division plane (2).

Presumably, minicell divisions and "normal" divisions share a similar pathway for the subsequent reasons: 1) incorporation of lon

into the minicell forming strain of Escherichia coli with the subsequent exposure to radiation (a condition known to prevent further "normal" division) also obviates minicell formation, 2) a double mutant, Escherichia coli Div 124ts, between the conditional division mutant, BUG 6 (79) and a minicell forming strain produces an organism which yields minicells only under conditions allowing division in BUG 6 (43), 3) a hybrid mutant (DZ 134) containing div 52ts (a cell division initiation mutant (109), and min mutant which affects the choice of division sites, fails to produce "normal" or minicell divisions at the restrictive temperature (110).

Not only are minicell divisions and "normal" divisions governed by a common influence but it seems possible that minicell site selection, div 52-ts division initiation, and BUG 6 cross wall elongation share a like pathway (110). Furthermore, a hybrid mutant, div 52-ts, a division initiation mutant and div 27ts, a dnaB mutant which divides in the absence of DNA synthesis, continued to divide after shifting to the nonpermissive temperature which suggests that the chromosome termination signal and div 52-ts division initiation cue act on a single membrane site that is altered in div 27ts strains (110).

These abnormal division sites which give rise to minicells appear related to the completion of DNA replication and/or segregation of the nuclear bodies. In mutant Bacillus subtilis div 355ts (cell division initiation mutant), upon shifting to the restrictive temperature filamentation occurs, although DNA synthesis proceeds normally and genomes are irregularly segregated along the filament length; returning to the permissive temperature results in division resuming producing

unequal, "normal"-size cells with an occasional small, anucleate cell being formed as an outcome of multiple cross walls in close proximity. The renewal of division is sensitive to protein inhibition (62).

Bacillus subtilis 168-151 ts, a presumed dnaB initiation mutant, exhibits loss of regulation of division site when grown at the restrictive temperature (60); this phenomenon is also true for Escherichia coli MX74T2ts27, a DNA mutant (37).

Mutants Used in This Study

A DNA initiation mutant, Bacillus subtilis 168, 134ts, was transferred by transformation to a minicell producing strain, CU403 Div IV-B1, creating the combined mutant, CU403 Div IV-B1, 134ts in hopes of exposing the correlation between DNA synthesis and cell division.

The conditional lethal DNA initiation mutant characterized by Mendelson and Gross (64) continues protein and RNA synthesis at a normal rate for several hours when logarithmic phase cells are transferred from the permissive to the restrictive temperature whereas DNA synthesis proceeds at a normal rate for only 20-30 min and then is diminished markedly. This amount of DNA synthesis occurring prior to reduction is approximately the same which results from protein inhibition of the parent mutant strain. Mapping was shown by Gross, Karamata, and Hempstead (28) to be part of the B group located near leucine.

After nitrosoguanidine mutagenesis of Bacillus subtilis M11, minicell producing div IV-B1 was isolated and then conveyed to CU403 (to procure an isogenic background) creating CU403 div IV-B1 which is defective in the location of the division site along the cell length

(80). In the course of growth polar divisions give rise to anucleate minicells, production of which is influenced by growth stage and media composition (65). These minicells contain the same ratio of RNA to protein as found in the parent cells. These minicells are motile reflecting functional energy metabolism and division has been noted in the absence of growth (15, 66, 81). Co-transformation and PBS1 transduction have disclosed this minicell producing mutation to be closely linked to pheA.

MATERIALS AND METHODS

Media

Adsorption Media

Adsorption media (74) contains 1 gram (g) yeast extract, 4 g NaCl, 5 g K_2SO_4 , 1.5 g KH_2PO_4 , 3 g Na_2HPO_4 , 120 milligrams (mg) $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, and 10 mg $FeCl_3 \cdot 6H_2O$ in 1 liter of distilled water.

Sporulation Media

Sporulation media (70) is comprised of 120 ml 10X Spizizen salts, 30 ml 20% glucose, 6 ml $CaCl_2$ (0.73 mg/ml), 2.4 ml $MgCl_2$ (20 mg/ml), 3 ml histidine (40 mg/ml), 12 ml thymine (2 mg/ml), 6 g potato extract, and 48 g agar in 1200 ml distilled water.

Spizizen Salts

Forty g NH_4SO_4 , 368 g $K_2HPO_4 \cdot 3H_2O$, 120 g KH_2PO_4 , 20 g sodium citrate and 4 g $MgSO_3 \cdot 7H_2O$ in 2 liters distilled water makes a 10X concentration of Spizizen salts (1).

Modified Spizizen 1

Modified Spizizen 1 (1, 89) contains 2 ml 20% glucose, 1 ml 10% yeast extract, 0.2 ml 10% casein hydrolysate, 2.5 ml 2 mg/ml thymine (auxotrophic requirement), and 2.5 ml 2 mg/ml histidine in 100 ml of 1X Spizizen salts.

Modified Spizizen 2

Modified Spizizen 2 (1) includes 10 ml Spizizen salts, 2 ml 20% glucose, 1 ml 0.05 M CaCl_2 , 2.5 ml 0.1 M MgCl_2 , 0.5 ml 1000 $\mu\text{M}/\text{ml}$ MgSO_4 , 0.1 ml 10% casein hydrolysate, 0.1 ml 10% yeast extract, 2.5 ml 2 mg/ml histidine, and 0.3 ml 2 mg/ml thymine (auxotrophic requirement) in 100 ml Spizizen salts.

Potato Agar

Boil for 5 min 200 g freshly diced potato in 1 liter of distilled water, filter, add 20 g tryptone, 2 g yeast extract, adjust pH to 7.2, add 1 liter distilled water, 30 g agar, and autoclave. Finally add 20 ml of 2 mg/ml thymine.

Schaeffer's Agar

Schaeffer's agar (52) contains 8 g nutrient broth, 1 g KCl , 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 17 g agar, adjust pH to 7, add 20 $\mu\text{g}/\text{ml}$ thymine, 5×10^{-4} CaCl_2 , and 1×10^{-6} FeCl_2 in 1 liter distilled water.

Minimal Media

Minimal media contains 100 ml 10X Spizizen salts, 25 ml 20% glucose, and 10 ml of auxotrophic requirement(s) at 2 mg/ml in 1 liter distilled water.

Complex Media

Complex media comprise Penassay Broth (PAB), Trypticase Soy Broth (TSB), and Tryptose Blood Agar Base (TBAB).

Maintenance

Strains were transferred weekly to TBAB containing thymine and incubated at room temperature (RT).

Isolation of Deoxyribonucleic Acid

Donor cells were grown overnight at 30 C, 100 RPM, in Penassay Broth (PAB) plus thymine (20 µg/ml). The culture was centrifuged in a Sorvall Superspeed RC 2-B at 9770 x g for 20 min and resuspended in 1/10 volume of 0.15 M sodium chloride--0.05 M sodium citrate buffer. Two hundred µg/ml of lysozyme (83) was added and incubated one hour at 45 C. Next 50 µg/ml of freshly prepared trypsin was added and further incubated for one hour at 45 C.

The treated culture was slowly added to about 4 volumes of ice cold 95% ethanol at which time the DNA fibers were spun out on a hooked glass rod and allowed to air dry for 15 min prior to suspending in 3 ml of 2 M NaCl--0.05 M Na Citrate buffer. Approximately 3 drops of chloroform were added to the buffered DNA and allowed to remain about 20 min then it was eliminated by bubbling. A sterility check was done (7, 22).

Transformation

The recipient cells were streaked onto TBAB plus thymine and grown overnight at 30 C. At 5:00 P.M. 10 ml of Spizizen 1 in an 18 mm x 17 cm test tube was inoculated with a small amount of the culture and left standing at RT without shaking overnight.

The subsequent morning the culture was transferred to a side arm flask and incubated at 30 C for 4-1/2 h, 250 RPM (Aquatherm Water Bath Shaker, New Brunswick Scientific Supplies). Next the culture was

diluted 1:10 into Spizizen 2 and incubated with shaking for one hour. At this point both DNA and cell controls were done.

One ml of donor DNA (~10 µg/ml) was added and the mixture incubated two hours at 30 C with shaking, 250 RPM. One-tenth ml samples of the undiluted culture was plated onto the appropriate selection medium and incubated at 30 C for 2-3 days. In this case minimal plates (1X Spizizen salts plus 0.5% glucose) plus 20 µg/ml thymine, either selecting for met⁺ or ilv⁺ depending as to whether CU 403 Div IV-B1 or CU 403 Div IV-A1, respectively, was used as the recipient.

Thousands of transformants were picked with sterile toothpicks and transferred to the appropriate minimal plate to allow growth and then replica plated at 30 C and 45 C. Colonies appearing at 30 C but not at 45 C were transferred with sterile toothpicks to TBAB plus thymine plates to grow and then replica plated at 30 C and 45 C. Colonies arising at 30 C but not 45 C were checked for purity and the proper markers followed by additional tests to confirm that the dnaB locus of 134ts along with the auxotrophic indicator marker were in fact transferred to CU 403 Div IV-A1/B1 by congression (1, 78, 89, 104, 105, 106, 107).

Spores

Potato Agar, Schaeffer's (52), and O'Sullivan and Sueoka's Media (70) were used in an attempt to produce an acceptable yield of spores; however O'Sullivan and Sueoka's method became the one of choice. Using this procedure, the cells were grown overnight at 30 C, 100 RPM, in Spizizen 1X media plus 0.5% glucose plus 20 µg/ml thymine. The

following morning the overnight culture was diluted 20 times into liquid sporulation media and grown to an OD at 660 nm of 1.4 then 0.5 ml samples were spread on sporulation agar and incubated at 30 C for 15-20 days.

Spores were collected with sterile distilled water, treated with 1% lysozyme, and incubated one hour at 45 C with shaking. One percent sodium lauryl sulfate was added and further incubated at 37 C with shaking for 30 min. The treated spores were centrifuged for 10 min at 9770 x g and resuspended in sterile distilled water and washed and centrifuged until the sodium lauryl sulfate was eliminated (4).

Spores were heat shocked at 80 C for about 5 min after which they were stored at 4 C in sterile distilled water.

Kinetics of DNA Replication

Measurement of DNA synthesis was determined by incorporation of tritiated thymine (5 μ Ci/ml) into macromolecules insoluble in cold 7% trichloroacetic acid (TCA).

A small sample of the test culture from an overnight TBAB plate was inoculated into 20 ml of Trypticase Soy Broth (TSB) containing 5 μ Ci/ml radioactive thymine and 2 μ g/ml of nonradioactive thymine and incubated at 30 C with shaking until the OD was approximately 0.1 at which time a 0.1 ml sample was pipetted into 8 ml cold TCA and the culture was concominantly halved, one portion continuing at 30 C and the remainder being shifted to 45 C. Samples were withdrawn and treated as described at periodic intervals for at least 3 h succeeding the split.

The samples were left overnight at 4 C and the next morning they were filtered onto 0.45 μ m HA Millipore filters, washed twice with cold 7% TCA, and placed in scintillation vials to dry overnight.

Ten ml of fluor consisting of 2 g PPO (2,5 diphenyloxazole) and 0.5 g POPOP ([1,4-bis 2-(5-phenyloxazole) benzene] per liter of toluene were added to each vial and the samples counted in a Packard Scintillation Counter (S. I. Coyne, University of Alabama Medical School at Birmingham, Department of Microbiology, and S. Keener, The University of Arizona, Department of Bacteriology, personal communication).

Autoradiography

Preparing the Slides

Microscope slides were soaked in distilled water overnight and the next morning rinsed with 50% alcohol. According to Caro's procedure (9, 10) the slides were subbed with 0.01% chromium potassium sulfate and 0.1% gelatin.

TSB containing 100 μ Ci/ml tritiated thymine and 2 μ g/ml of non-radioactive thymine was inoculated with an overnight TBAB culture of the test strain and grown with shaking at 30 C until an OD of 0.1 was reached. A 0.5 ml sample was pipetted into 0.5 ml of 7% TCA containing 1 drop 3.6% formalin. The culture was halved, one part remaining at 30 C and the other shifted to 45 C. Samples were periodically withdrawn and handled in the same manner as previously described until at least 3 hours subsequent to the split.

The samples were left overnight at 4 C and the succeeding morning were filtered through a 13 mm HA filter (0.45 μ m) in a Swinney

apparatus and washed twice with distilled water and resuspended in approximately 1 ml of distilled water. About 2-3 drops of the suspension were smeared on a subbed slide and observed for cell concentration (oil immersion, Wild Heerbrugg M-20 phase contrast microscope--Switzerland). Five to 8 slides per sample were prepared this way and dried on a hot box, 55-60 C. The slides were then washed for 10 min with cold 5% TCA and rinsed twice with cold distilled water for 2 min each time using staining jars. After drying they were coated with Ilford L-4 Nuclear Research Emulsion prepared as follows: in the dark-room, using a safelight, 30 g of emulsion was weighed and melted in 20 ml of distilled water in a 45 C water bath.

Each slide was dipped in the emulsion at 40 C and dried vertically for 2 hours then placed in a slide box to self-expose until the time to develop.

Developing the Autoradiography Slides

After 4 days the slides were developed according to the following protocol maintaining all solutions at $20\text{ C} \pm 1\text{ C}$: Developer (Kodak-19), 4-5 min, Kodak Stop Bath, 10 sec, Fixer (F-24: 240 g sodium thiosulfate, 10 g anhydrous sodium sulfate, and 25 g sodium bisulfite dissolved in 500 ml distilled water at 52 C after which 500 ml distilled water at RT is added), 5 min, and washed in distilled water for 5 min.

The slides were dried and stored in slide boxes. For examination, two drops of distilled water were used for mounting the cover-slip.

Photographing the Autoradiography Slides

The slides were focused on oil immersion using a phase contrast microscope and photographed (Canon, Ftb). The film was developed using Microdol, 9 min, stop bath (30 sec), and Fixer (4 min), and washed in tap water for 1 h followed by a 5 min rinse in distilled water and hung to dry.

Prints were made using an Omega 83 enlarger and Medalex No. F-3 paper. Kodak Dektol was used for developing, Kodak stop bath, Kodak fixer, and the water rinse. The prints were dried on the usual print drying apparatus.

Transduction

Lysate Preparation

High Titer Stock PBS1 Lysate. A loopful of Bacillus pumilus culture grown overnight at 30 C on a TBAB plate was added to 25 ml PAB and grown at 30 C with shaking for 4 h. In the meantime 0.1 ml of stock PBS1 was pipetted into 1.3 ml adsorption media. Two-tenths ml of motile Bacillus pumilus was added to each tube containing PBS1 plus adsorption media and incubated at RT for 5 min then 1.5 ml of 2.2% TBAB was added and overlayed on freshly prepared 3.3% TBAB plates and incubated at 30 C (22, 25, 36, 54, 99, 100).

In the morning 10 ml of adsorption media were added to each plate and left standing at RT for at least 3 h then scraped and centrifuged at 13,218 x g for 15 min. The supernatant fluid was filtered,

sterilized and the phage concentrated to approximately 10^{13} pfu/ml by centrifuging 2 h at $45,864 \times g$ (94, 95).

Donor CU 403 Div IV-B1 134 ts Lysate. A liquid lysate was made using CU 403 Div IV-B1, 134 ts as the donor strain by growing this culture overnight on a TBAB supplemented with thymine plate and in the morning transferring a loopful to 25 ml PAB with 20 $\mu\text{g/ml}$ thymine and growing 4 h with shaking at 30 C. Cells were checked for motility then 1 ml of PBS1 ($\sim 10^9$ pfu/ml) stock lysate was added and further incubated for 1 h. Chloramphenicol (CAM, 5 $\mu\text{g/ml}$) was added and the incubation continued overnight. The succeeding morning the culture was centrifuged at $9770 \times g$ for 20 min and the supernatant filter-sterilized using a 47 mm HA Millipore filter (0.45 μm).

Treatment of the Recipient

The recipient culture was grown overnight on a TBAB plus 20 $\mu\text{g/ml}$ uracil plate at 30 C. From this plate 2.5 ml PAB plus uracil was inoculated and the culture incubated 3 h at 30 C. After checking for motility, 2.5 ml of PBS1 stock lysate prepared on CU 403 Div IV-B1, 134 ts was added (moi ~ 1) and incubated 25 min at 30 C. Prior to adding the lysate, controls were done by plating recipient cells on appropriate plates to check for revertants and by streaking donor lysate for sterility.

The entire culture was transferred to a centrifuge tube and 3 ml of 0.85% NaCl was added and centrifuged at $9770 \times g$ for 5 min. The pellet was resuspended in 0.5 ml 0.85% NaCl which was plated on appropriate plates and incubated for 2-3 days at 30 C.

Transductants were transferred to minimal plates with sterile toothpicks and allowed to grow, then replica plated at 30 C and 45 C. Colonies appearing at 30 C but not 45 C were transferred to TBAB plus uracil plates and after growing, replica plated at 30 C and 45 C. All colonies arising at 30 C but not at 45 C were screened for morphology to determine how many of the temperature sensitive transductants were additionally CU 403 Div IV-B1.

Growth Chambers for Spores

Growth chambers for spores (commonly referred to as "chips") were constructed by layering 5-6 drops of 1.7% agar in TSB plus thymine onto a circular no. 2, 2 mm glass coverslip. After the media appeared opaque 1 scant drop of spore suspension (10^8 /ml) was gently dispersed over the agar and allowed to dry for 15 min at which time air pockets were created by piercing the agar with a sterile toothpick then another coverslip was placed on top and the "sandwich" sealed with a 0.25 x 4 inch strip of parafilm.

Clonal Growth Studies

At least two chips were placed in the 30 C incubator for approximately 5 h then transferred to a heated incubator stage (Reichert, Austria) maintained at 30 C for the necessary time followed by a shift to 45 C. This incubator was placed on a microscope (Wild, Switzerland) stage where the growth of the clone was observed and photographed (Kodak Panatomic film, ASA 32 in a Canon FT_b camera) every hour for at least 3 h.

Clones were followed at 30 C and at 30-45 C shifts. Outgrowth of spores at 45 C was attempted but these cells would generally lyse at about 15 μ m in length before one division.

The film was developed using Microdol 9 min, Stop bath 30 sec, Fixer 4 min, and tap water for 1 h.

Using an Airoview projector the film was projected onto paper where the clone was traced. The projector was calibrated showing 1 mm = 1 μ m. The traced clone was measured with a Minerva map measurer (Switzerland) and the results graphed for further statistical analysis.

Strains Used

Bacillus subtilis 168ts-151: tryptophan⁻ thymine⁻

Bacillus subtilis 168ts-134: tryptophan⁻ thymine⁻

Bacillus subtilis 168ts-75: tryptophan⁻ thymine⁻

Bacillus subtilis CU404 Div IV-A1: thyA⁻, thyB⁻, ilvD⁻

Bacillus subtilis CU403 Div IV-B1: thyA⁻, thyB⁻, metB⁻

Bacillus subtilis m-11: leuA⁻, ura⁻, metB⁻

RESULTS

DNA inhibitors, such as nalidixic acid, mitomycin C, ultraviolet light, and thymine starvation have been employed as means of acquiring information concerning the control of cell division. Often such chemical and physical inhibitors not only influence DNA synthesis but also affect cell division, per se. Therefore, caution must be exercised when using these agents. Consequently, it is preferable to use conditional lethal mutants which operate normally at the permissive temperature but malfunction at the restrictive temperature.

Hence, a DNA initiation mutant, Bacillus subtilis 168-134ts, was transferred to a minicell producing strain, CU 403 Div IV-B1, by transformation. Markers in the combined mutant were corroborated by transductional analysis. DNA replication kinetics, genome location by autoradiography, and clonal study of cell division patterns during spore outgrowths were investigated.

Construction and Confirmation of the Combined Mutant

Transformation (congression) was employed for construction of the combined mutant which involved picking and screening thousands of transformants, with the resultant isolation of 8 CU 403 Div IV-A1, 151ts and 9 CU 403 Div IV-B1, 134ts combined mutants (Figure 1). These mutants grew at 30 C on complex plates (TBAB) but formed only a haze when grown at 45 C for 2 h, at which time the colonies were observed under oil immersion with a phase contrast microscope and the cells

Figure 1. Outline of construction of Bacillus subtilis CU 403 DIV IV B1, 134ts.

appeared contorted with lemon-shaped swellings located primarily sub-polarly and centrally (Figure 2).

To verify integration of the dnaB defective marker, transductional analysis was performed (Table 1). Using PBS1 phage, a lysate was made on the donor, the combined mutant, and conveyed to M-11, selecting for leu⁺ and met⁺ transductants. One notes that (Figures 3 and 4) dnaB is located in the proximity of leuA as well as Div IV-B1 (near 7:30), whereas metB is positioned around eleven o'clock. An equal number of transductants from metB⁺ and leuA⁺ minimal plates were picked and replica plated in minimal plates at 30 and 45 C. One would anticipate a greater number of temperature-sensitive (ts) colonies on the leu plates and such was the case. All ts mutants were grown at 30 C on complex media and then replica plated on complex at 30 and 45 C. Sixty-three of the 124 leuA⁺ mutants were ts, resulting in a 50.8% cotransduction frequency compared to 0.8% for metB⁺ since only 1 per 124 of metB⁺ was additionally ts. Of the 63 leuA⁺, ts transductants 54 were also Div IV-B1 resulting in an 85.7% cotransduction; about 35% of the leuA⁺, ts transductants failed to incorporate the additional Div IV-B1 fragment even though about 6% of the donor genome is transferred to the recipient during PBS1 transduction. These results confirm that the dnaB initiation marker was in fact integrated at the correct site in the minicell producing strain's genome.

DNA Replication Kinetics

Difficulty was encountered in acquiring a good yield of spores which was primarily the determining factor in selecting one of the 17

Figure 2. Swellings In A Culture of CU 403 Div IV-B1, 134ts.

This culture was grown on a TBAB plus thymine plate at 30 C and the following morning a loopful was transferred to a fresh TBAB plus thymine plate and incubated at 45 C for 2 h at which time a photograph was taken with a phase contrast microscope on oil immersion.

Table 1. Transduction of DIV IV-B1 and 134ts from CU 403 Div IV-B1, 134ts into m-11, metB⁻, ura⁻, leuA⁻.

A. Selection For:	Number Transductants	Number of <u>ts</u>	% Cotransductants
<u>metB⁺</u>	124	1	0.8
<u>leuA⁺</u>	124	63	50.8
B. <u>leuA⁺, ts</u> Transductants	Number of <u>Div IV-B1</u>	% Contrtransduction <u>ts, Div IV-B1</u>	
63	54	85.7

Figure 3. Genetic Map of *Bacillus subtilis*.

Courtesy of Young and Wilson (108, pp. 90-91).

Figure 4. Revised Genetic Map of *Bacillus subtilis*.

Courtesy of Lepesant-Kejzlaroua et al. (53, p. 833).

combined mutants for further study. Nevertheless, 15 strains were subjected to DNA replication kinetics analysis (Figures 5 and 6), revealing that following transfer to the restrictive temperature turn off of DNA synthesis ranged from 45 min to 1-1/2 h with an increase of 2-1/2 to 13 fold.

CU 403 Div IV-B1, 134ts became the mutant of choice for additional study. Thus further comments will pertain specifically to this strain. In Figure 7 it is noted that DNA synthesis is turned off 1-1/2 h after transfer to 45 C, the restrictive temperature, with an 8-1/2 fold increase whereas at 30 C, the permissive temperature, DNA replication is not retarded. Light scattering indicates mass increase continues for 3 h with an increment of eightfold at 45 C but proceeds in an unaffected manner at 30 C. After approximately 3 h at 45 C, the cells begin lysing and by 3-1/2 h cellular debris is predominantly observed, but at 30 C the cells remain intact.

The large increase in DNA synthesis following transfer to the restrictive temperature might be explained in terms of multifork replication occurring during growth at 30 C and also leakiness of the dnaB marker in the new genetic background.

The control, CU 403 Div IV-B1 (Figure 8), synthesizes DNA for 3-1/2 hours after transfer with an increase of 13 fold whereas cell mass was continuing to increase at 4 h.

Figure 9 portrays a plot of OD_{660 nm} versus CPM (DNA) in the combined mutant and control, the minicell producing strain, both grown at 30 and 45 C. The ratios are quite similar until reaching an OD of 0.33 at which time differences are perceived. For instance, the control

Figure 5. O.D. and C.P.M. Plotted as A Function of Time in Six (A → F) CU 403 Div IV-B1, 134ts Mutants.

Each culture is growing logarithmically in TSB containing 5 $\mu\text{Ci/ml}$ H^3 -thymine and 2 $\mu\text{g/ml}$ thymine until 0 time (O.D. ~ 0.1) when the culture was halved, one portion continuing at 30 C and the remainder shifted to 45 C. (O.D.: $\square$, 30 C; $\blacksquare$, 45 C and C.P.M.: $\circ$, 30 C; $\bullet$, 45 C.)

Figure 6. O.D. and C.P.M. Plotted As A Function of Time In Eight (A $\rightarrow$ H) CU 403 Div IV-A1, 151ts Mutants.

Each mutant is growing exponentially in TSB containing 5 μ Ci/ml H^3 -thymine and 2 μ g/ml thymine until 0 time (~ 0.1 O.D.) when the culture was split, one-half continuing at 30 C and the remainder shifted to 45 C. (O.D.: $\square$, 30 C; $\boxtimes$, 45 C and C.P.M.: $\bullet$, 30 C; $\star$, 45 C.)

Figure 6, Continued.

Figure 7. O.D. and C.P.M. Plotted As A Function of Time In
CU 403 Div IV-B1, 134ts.

This culture is growing exponentially in TSB containing 5 $\mu\text{Ci/ml}$ H^3 -thymine and 2 $\mu\text{g/ml}$ thymine at 30 C until 0 time when the culture was halved, one portion continuing at 30 C and the remainder shifted to 45 C.

Figure 8. O.D. and C.P.M. Plotted As A Function of Time In CU 403 Div IV-B1.

This culture is growing exponentially at 30 C in TSB containing 5 μ Ci/ml H^3 -thymine and 2 μ g/ml thymine until 0 time when the culture was halved, one portion continuing at 30 C and the remainder shifted to 45 C.

Figure 9. C.P.M. Plotted As A Function of O.D._{660nm} in CU 403 Div IV-B1 and in CU 403 Div IV-B1, 134ts.

Each culture is growing logarithmically at 30 C in TSB containing 5 μ Ci/ml H^3 -thymine plus 2 μ g/ml thymine until an O.D. ~ 0.1 when the culture was split, one-half continuing at 30 C and the other shifted to 45 C.

Figure 10. C.P.M./O.D. Plotted As A function of O.D. In CU 403 Div IV-B1 and In CU 403 Div IV-B1,
134ts.

Each culture is growing exponentially at 30 C in TSB containing 5 $\mu\text{Ci/ml}$ H^2 -thymine and 2 $\mu\text{g/ml}$ thymine until an O.D. ~ 0.1 when the culture was halved, one part continuing at 30 C and the remainder shifted to 45 C.

Figure 10. C.P.M./O.D. Plotted As A Function of O.D. In CU 403 Div IV-B1 and In CU 403 Div IV-B1, 134ts.

continues to be comparative at 30 C and 45 C with the combined mutant at 30 C, however, at 45 C in the combined mutant the CPM versus OD levels off. There exists a disparity of 3.8×10^4 CPM at an OD of 0.9 between the double mutant at 30 and 45 C. However, of importance is that there is a discrepancy of 2.6×10^4 CPM at an OD of 0.9 between the double mutant and the control both at 45 C. In other words, the control, which is presumably isogenic with the double mutant, except for the absence of the defective dnaB initiation marker, and the double mutant behave in a like fashion at 30 C; whereas at 45 C the influence of the defective dnaB marker is observed in the double mutant accounting for the marked difference between the two.

Figure 10 substantiates the trends previously mentioned such that occurring at an OD of approximately 0.33 there exists a conspicuous deviation in the direction which the combined mutant at 30 C and 45 C and the combined mutant and control both at 45 C take. At 45 C in the combined mutant the ratio of CPM/OD versus OD drops, noting a difference of 4.5 compared to the double mutant at 30 C and of 3.2 contrasted to the control at 45 C at an OD of 0.9. These graphs depict the influence of the deficient dnaB marker on behavior patterns of the minicell producing strain.

Autoradiographic Inspection

To further establish incorporation of the dnaB marker, autoradiographic studies were done according to Caro's procedure (9, 10) (Table 2). A log phase culture grown at 30 C in TSB containing 100 $\mu\text{Ci/ml}$ of tritiated thymine and 2 $\mu\text{g/ml}$ of nonradioactive thymine was halved

Table 2. Autoradiography of H^3 -thymine incorporation into DNA of
CU 403 Div IV-B1, 134ts.

Time ^a (Minutes)	O.D. 30 C	660 nm 45 C	% Cell Length Covered By DNA Grain Clusters	
			30 C	45 C
0	.15	-	54.5(13.48) ^b	
30	.23	.37	37.0(9.29)	35.2(9.39)
120	.56	.8	60.2(8.95)	8.6(4.51)
240	.9	.39	45.6(9.77)	5.8(3.19)

^aAn exponential culture in TSB containing 100 μ Ci/ml of H^3 -thymine plus 2 μ g/ml thymine growing at 30 C was halved at an O.D. of 0.15 at which time (time 0) one portion was transferred to 45 C and the remainder continued at 30 C.

^bStandard deviation.

at an OD of 0.15, one portion continuing at 30 C and the remainder shifted to 45 C. At time 0, when the culture was halved, 54.5% of the cell length was covered with DNA grain clusters (determined by measuring length of DNA grain clusters compared to measured length of cell) whereas 120 min later at 45 C only 8.6% coverage was observed compared to 60.2% at 30 C. The extreme reduction of DNA replication can thereby be inferred in addition to the occurrence of a significant increase in cell mass in the absence of DNA synthesis at 45 C. The time at which this impressive contrast between 30 and 45 is noted corresponds nicely to the DNA replication kinetic curve disclosing abatement at approximately 1-1/2 h (Figure 7).

Photographs (Figure 11) of the autoradiographs at the aforementioned, designated times show that at time 0 DNA is distributed along the cell length barring the polar regions and minicells which lack detectible nuclear material. At 30 min, 30 and 45 slides appear quite similar but with the DNA grains more dispersed than at 0 time. At 30 C in 1-1/2 h the DNA is heavily distributed throughout the central areas of the cells excluding the poles, whereas at 45 C the cells are deficient in discernible divisions along their extraordinary length and few DNA grain clusters are perceived.

Growth and Cell Division Studies

Growth chambers were utilized to examine cell division processes in 232 cells comprising 23 clones at 30 C compared to 1156 cells in 97 clones grown at 30 C and shifted to 45 C. Clones were photographed hourly, the negatives projected onto paper and the clonal images traced,

Figure 11. Photograph of Autoradiography of H^3 -Thymine Incorporation Into DNA of CU 403 Div IV-B1, 134ts.

A logarithmic phase culture in TSB containing 100 $\mu\text{Ci/ml}$ thymine plus 2 $\mu\text{g/ml}$ thymine growing at 30 C was halved at an O.D. ~ 0.1 at which time (time 0) one portion was transferred to 45 C and the remainder continued at 30 C.

and the tracings were measured noting lengths to each septum from one end and total lengths of clones, then the results finally graphed (Figures 12 and 13).

Probability of a Minicell Division

At 30 C (Figure 14), 78 minicell divisions occurred in a total of 177 divisions resulting in a 0.44 probability of a minicell division or 2.3 divisions being required to produce one minicell; at 45 C 393 minicell divisions took place out of 972 divisions constituting a 0.40 probability of a minicell division or 2.5 divisions giving rise to 1 minicell.

Table 3 portrays the frequency of clones producing a certain number of minicell divisions and total divisions at various times. It is observed that 7-8 h succeeding germination both the greatest number of minicell divisions and total divisions take place. Most minicell divisions occur after 2 h following transfer to 45 C after which commencement of lysis becomes apparent.

The frequency of minicell divisions occurring at the specified probabilities is shown in Figure 15. The highest frequency takes place at a probability of 0.41 to 0.5. In part the numbers of minicell divisions constituting a probability of 1.0 can be attributable to clones in which the first division is a minicell producing division.

From plotting the probability of a minicell division as a function of clonal length (Figure 16), it was hoped to ascertain whether the chance of a minicell division varied with growth. Once again it is noted a probability of 1.0 is predominant at shorter lengths where the

Figure 12. Spore Outgrowth of CU 403 Div IV-B1, 134ts Germinated and Grown at 30 C in TSA (1.7% Agar + 2 μ g/ml Thymine) for 5 h At Which Time the Temperature Was Increased to 45 C.

Photographs 3, 4, and 5 represent 1, 2, and 3 h respectively succeeding temperature increase.

Figure 12, Continued.

Figure 13. Clonal Length and Number of Divisions As A Function of Time of CU 403 Div IV-B1, 134ts Spore Outgrowth.

Spores were germinated and grown at 30 C on TSA plus thymine for 5 h at which time the temperature was increased to 45 C (time 2). The horizontal lines represent clonal lengths whereas the vertical lines depict divisions along the clonal length. Times 3, 4, and 5 represent 1, 2, and 3 h subsequent to the temperature shift.

$$p = \frac{\text{minicell divisions}}{\text{total cell divisions}}$$

1. CU 403 Div IV B1, 134ts at 30 C

$$P = \frac{78}{174}$$

= 0.44 (2.3 divisions required to produce 1 minicell)

2. CU 403 Div IV B1, 134ts 30 → 45 C transfer

$$P = \frac{393}{947}$$

= 0.40 (2.5 divisions required to produce 1 minicell)

Figure 14. Probability of a Minicell Division.

Table 3. Number of clones with total divisions and number of clones with minicell divisions at designated times following spore germination at 30 C of CU 403 Div IV B-1, 134ts.

Shift to 45 C occurred 5 h following germination.

Figure 15. Probability of A Minicell Division Plotted As A Function of the Frequency of the Probability of Minicell Divisions in Clones of CU 403 Div IV-B1, 134ts.

Results based upon spores germinated and grown on TSA with thymine at 30 C for 5 h at which time the temperature was increased to 45 C.

Figure 16. Probability of Minicell Divisions/Total Divisions Plotted As A Function of Clonal Length in CU 403 Div IV-B1, 134ts.

Spores were germinated and grown at 30 C on TSA with thymine for 5 h at which time the temperature was increased to 45 C. These parameters represent data collected following the shift.

first and only division gives rise to a minicell. With increased length in the absence of DNA synthesis, if the expectation of a minicell division increased, this might be related to lack of DNA in those regions where the minicell divisions would occur. However, it would be necessary to assume minicell production is not initially under DNA regulation. Thus, the graph merely reflects that a prescribed probability of a minicell division does not take place at one designated length. In addition, as the lengths become longer there is greater randomization of the probability of a minicell division.

Time-Length Correlation

Figure 17 represents clonal length correlated with time. It is observed one hour following transfer to 45 C most of the clones are less than 251 μm , whereas at times 2 and 3 h there exists more dispersion and, also, fewer clones are plotted for times 3, 4, and 5, the reason being that after 2 to 2-1/2 h many of the clones began lysing. Thus, what one notes after 2 h is not an accurate portrayal of the time-length relationship. One can merely say these figures illustrate the existence of clones growing at different rates at a specific time.

Figure 18, clonal length $\text{time}_x / \text{clonal length time}_0$ plotted as a function of time, repeatedly demonstrates that after 2 h few clones are plotted due to lysing and that at time 1 h most of the clones have increased in the realm of 1-4 times whereas by time 2 h more variation is seen. Once again it is observed that dissimilar rates of clonal growth are prevalent at the prescribed times.

Figure 17. Clonal Length Plotted As A Function of Time in CU 403 Div IV-B1, 134ts.

Data based upon spores germinated and grown on TSA with thymine at 30 C for 5 h at which time the temperature was increased to 45 C. These times represent the period subsequent to the temperature increase.

Figure 18. The Ratio of $\text{Clonal Length}_{\text{Time } 0} / \text{Clonal Length}_{\text{Time } x}$ Plotted As A Function of Time in CU 403 Div IV B-1, 134ts.

Data are based upon spores germinated and grown on TSB with thymine at 30 C for 5 h at which time the temperature was increased to 45 C. Time is in hours succeeding temperature increase.

Division Suppression

Linear Regression Analysis for Divisions as a Function of Time.

The inclination of the slope is related to the degree of division suppression for as the slope increases greater length is required for a division (Figures 19, 20, 21, 22). Consequently, of the above specified strains (Table 4) CU 403 at 30 C is the least suppressed (more divisions at shorter lengths) and CU 403 Div IV-A1 the most subdued (fewest divisions as a function of length) (58).

CU 403 Div IV-B1 and the combined mutant at 30 C (Figure 20) have similar slopes of 20.8 and 19.8 respectively whereas the combined mutant at 45 C (Figure 21) has a slope of 24.9 reflecting greater suppression most likely due to the presence of the defective dnaB marker. In other words at 30 C CU 403 Div IV-B1 and the double mutant display a comparable number of divisions for a designated length whereas at 45 C the double mutant demonstrates considerable fewer septations at that same length presumably due to the existence of the mutant dnaB marker.

Percent Division Equivalents Expressed. Calculation of percent division equivalents expressed is derived by comparing the observed number of divisions in a clone of a specific length (experimental) to the expected number of divisions in a clone of that same length (control) and calculating what percent the observed is of the expected. This percent division equivalents expressed was ascertained for CU 403 Div IV-B1, 134ts at 30 C and 45 C based upon CU 403 (wt) at 30 C and 45 C and upon CU 403 Div IV-B1 at 30 C (Table 5).

Figure 19. Clonal Length Plotted As A Function of Divisions In CU 403.

Spores were germinated and grown on TSA with thymine at 30 C for 5 h at which time the temperature was increased to 45 C. (Based on data collected following temperature increase.)

Figure 20. Clonal Length Plotted As A Function of Divisions In
CU 403 Div IV-B1, 134ts, 30 C.

Spores were germinated and grown on TSA with thymine at 30 C.

Figure 21. Clonal Length Plotted As A Function of Divisions In
CU 403 Div IV B-1, 134ts, 45 C.

Spores were germinated and grown on TSA with thymine at 30 C for 5 h at which time the temperature was shifted to 45 C. (Based on data collected after the shift.)

Figure 22. Comparison of Clonal Lengths Plotted As A Function of Divisions.

A, CU 403₃₀ C; B, CU 403₄₅ C; C, CU 403 Div IV B-1₃₀ C; D, CU 403 Div IV-B1, 134ts₃₀ C; E, CU 403 Div IV-B1, 134ts₄₅ C; and F, CU 403 Div IV-A1₃₀ C.

Table 4. Summary of linear regression results.

Strain	Temperature (°C)	Intercept	Slope
<u>CU 403^a</u>	30	17.6	8.8
<u>CU 403</u>	45	9.41	17.09
<u>CU 403 Div IV-A1^a</u>	30	44.6	26.4
<u>CU 403 Div IV-B1^a</u>	30	27.04	20.8
<u>CU 403 Div IV-B1, 134ts</u>	30	72.7	19.79
<u>CU 403 Div IV-B1, 134ts</u>	45	71.99	24.9

^aMendelson and Coyne (63).

Table 5. Calculation of percent division equivalents expressed.

CU 403 Div IV-B1, 134ts Grown At	Ob- served	Based On					
		Expected D.E. wt _{30 C} = $\frac{\mu m - 17.6}{8.8}$	%	Expected D.E. wt _{45 C} = $\frac{\mu m - 9.41}{17.09}$	%	Expected D.E. B1 _{30 C} = $\frac{\mu m - 27.04}{20.8}$	%
30 C	177	733	24.1	402.89	43.9	279.8	63.3
45 C	972	3813.8	25.5	2041.06	47.6	1545.3	62.9

Seemingly, there is little difference in the combined mutant's behavior at 30 C and 45 C for at both temperatures when compared to CU 403 at 30 C it showed 24.1 and 25.5% division equivalents expressed, respectively, or in other words division occurs only one-fourth as often as expected whereas similarities in the combined mutant at 30 and 45 C are once again observed when related to CU 403 at 45 C thereby demonstrating percent division equivalent expressed of 43.9 at 30 C and 47.6 at 45 C or the combined mutant is dividing about one-half as frequently as anticipated; based upon CU 403 Div IV-B1 at 30 C, 63.3% division equivalent expressed is noted at 30 C and 62.9% at 45 C in the combined mutant. Succinctly, less suppression is taking place compared to CU 403 Div IV-B1 as contrasted to CU 403; furthermore, 38.75% division equivalent expressed was determined for CU 403 Div IV-B1 based upon CU 403 at 30 C (15) which is about 15% less than that found for the double mutant.

DISCUSSION

DNA Replication, Protein Synthesis, and Cell Division Interrelationships

It is speculated (11, 12, 13, 41, 82) that DNA synthesis and cell division are coordinated to insure that each daughter cell contains a copy of the parental nuclear material providing for the preservation and continuation of the clone. Nevertheless, it remains uncertain how this relationship is intertwined. Evidence exists substantiating the interdependence of the association, but exceptions have been noted whereby cells continue to divide in the absence of DNA replication (21, 24, 37, 38, 90) which prevents a concise interpretation of the related processes.

In Escherichia coli B/r at 37 C with generation times of 20 to 60 min, it has been established that chromosome replication remains constant requiring about 40 min with division normally following in 20 to 25 min (11, 12, 30, 31) irrespective of the growth rate (13, 30, 31).

Pierucci (72) has shown in experiments with E. coli B/r that at all temperatures and in all media the division interval was $1/2$ the replication time ($D = 1/2 C$) suggesting that a common mechanism controls chromosomal replication and the progression of the cell toward division after completion of a round of replication (85). Pierucci and Helmstetter (73) also maintain that the time between divisions, the time for a round of replication (C), and the time between completion of a round and cell division (D) were threefold longer at 21 C than at 37 C in cultures

with generation times of 60 to 120 min. In contrast Kubitschek and Freedman (48) profess that $C + D$ are constant in exponentially growing cultures with generation times as long as 120 min.

Factors which inhibit DNA synthesis, such as nalidixic acid, ultraviolet irradiation, mitomycin C, thymine starvation, and growth of temperature-sensitive mutants at the restrictive temperature, generally elicit filament formation (17, 45, 87). Yet various and diverse agents and temperature-sensitive mutants causing production of filaments do not alter DNA synthesis, thus possibly affirming dependence of cell division on DNA synthesis rather than the converse (8, 62, 88).

Furthermore, to assure perpetuation of the clone, it is favorable that sequential processes occur to furnish each daughter cell with a duplicate of parental nuclear material, thus once again connoting that in some fashion DNA replication is accountable for the regulation of cellular events instrumental in the timing of cell division.

Endeavors to disentangle the intricacies of DNA synthesis, protein synthesis, and cell division continues to be incomplete, but ground has been gained, as it is known that protein synthesis but not DNA replication is required for initiation of DNA replication (49, 73) which occurs upon attainment of a critical size per chromosome origin (16) and once inaugurated DNA replication can proceed in the absence of protein synthesis. Once a round of replication has taken place, cell division can occur even when synthesis of DNA is inhibited (11, 12, 31); significant is that cell division is contingent upon the termination of chromosome replication ostensibly coding for the necessary termination protein/s (11, 12, 31, 43, 57). Apparently protein synthesis commencing

at the time of DNA initiation and persisting concomitantly with DNA synthesis is imperative for the impending cell division (73, 88). If protein synthesis is obviated following chromosome replication, division will still take place within the usual 20 to 25 min indicating the essential proteins have been produced and are merely assembled or function during this period. Inhibiting protein synthesis during the cycle prior to the termination of chromosome replication will prevent cell division.

Jones and Donachie's (43) investigations lend credibility not only to the gravity of termination of replication as a prerequisite for cell division but furthermore profess that the production of the termination protein/s induces dissociation of the DNA replication complex from the membrane thereby abolishing the block to division and possibly becoming the site where septum formation occurs (43).

Others have suggested that the chromosome replication complex is attached at the origin, replication point, and terminus to the cell membrane, thereby blocking division (41, 72, 77). In a resting state the chromosome is not connected to the membrane (41). Terminal division in Erwinia involving metabolites such as pantoyl lactone indicates a triggering action and subsequent septum formation which is not ascribed to protein or RNA synthesis (29).

If the block to division is instituted by the chromosome replication complex attaching to the cell membrane at the time of DNA initiation and remaining affixed until termination of the replication, untimely divisions might be prevented.

Regardless of the support favoring the interdependence of DNA synthesis and cell division, exceptions have been noted whereby

divisions pursue in the absence of DNA synthesis (33, 37, 38, 60, 62, 84, 85, 90, 91).

Abnormal Division Sites

Normally, division proceeds in an equatorial position resulting in two equal sized cells each containing a copy of parental genetic material. Yet in certain DNA mutants and minicell producing mutants the division sites are abnormally located (2, 3, 15, 26, 34, 35, 61, 80) as is the situation in the studied combined mutant, CU 403 Div IV-B1, 134ts. Minicells are produced by a mechanism which appears structurally similar to a normal division (3, 45) except in location giving rise to a longer than normal cell containing the nuclear material and a small, anucleate cell [in Bacillus subtilis (80)].

The means by which minicell formation is regulated remains obscure but considerable evidence exists suggesting that normal division (11, 45), minicell formation, cross wall elongation, and division initiation share a common division pathway (79, 109).

Adler et al. (1) found that the minicell producing division itself in Escherichia coli proceeded at a rate comparable with that observed for normal division however seemingly the nuclear region of the cell was not involved since DNA was not distributed to both sides of the division plane. Adler and Hardigree (2) have alleged that a mutation has occurred which interferes with the normal spatial relationship between some essential division triggering signal and the potential division planes of the cell.

In the minicell forming strains there may be secondary sites located near both poles which can also be activated to form crosswall and septum; it is possible these sites exist in normal cells but are blocked from responding to a division initiation stimulus (2, 71). Teather, Collins, and Donachie (96) claim that in minicell producing mutants potential division sites once formed are never inactivated. The cell poles as well as equatorial regions are available as potential sites and the probability of a division taking place at any of the potential division sites is equal, but only enough "division factor" is manufactured at each unit cell doubling (19) to allow a single division. Therefore, the production of a minicell division precludes a normal division elsewhere, causing a filamentous extension. However, experiments conducted by Zusman and Krotoski (110) with DZ134, a cell division initiation and minicell double mutant, refute the random selection of quiescent division sites for a minicell division, due to observance of minicells only at the older cell poles. Coyne and Mendelson (15) also claim that minicells are preferentially situated at the older poles.

Another explanation for minicell production is offered by Inouye from his investigations with a recA mutant of E. coli (38) and a DNA mutant, E. coli ts27 (37). He proclaims that septum formation in normal cells is usually blocked by a heat sensitive factor and only at the end of DNA replication is this barrier suppressed by an unknown mechanism. Possibly this block is inactivated by an unstable agent which is produced in a limited amount at the termination of replication. The minicell mutant might produce an excess amount of this inconstant agent so that extra septations occur (39, 40).

Discussion of Results

In the studies reported here, a combined DNA initiation and minicell producing mutant was constructed by transformation to study division patterns. Markers in the combined mutant were verified by transduction. DNA replication kinetics, genome location by autoradiography and clonal analysis of cell division behavior during spore outgrowths were investigated.

Transduction Analysis

A 50.8% cotransduction frequency for 134ts and leuA⁺ was found. Eight-five and 0.7 percent of these transductants were additionally div IV B1 thus confirming the integration of the ts, dnaB marker at the proper locus in the minicell producing strain's genome. The transductants which were leuA⁺, ts, and div IV-B1 manifested contorted filaments with lemon shaped swellings subpolarly and centrally when grown for 2 h at 45 C on complex media thereby offering further evidence for the presence of the dnaB defective marker (6, 64).

Kinetics of DNA Replication

DNA replication kinetics demonstrates the influence of the dnaB marker on the minicell producing strain. In the combined mutant, DNA synthesis curtailment occurs about 1-1/2 h after transfer to the restrictive temperature with an increase of 8-1/2 fold compared to the minicell producing strain increasing 13+ times before diminishment of DNA synthesis around 3-1/2 to 4 h. Cell mass, inferred from OD readings at 660 nm increased 8 fold in 3 h prior to cessation in the combined mutant, whereas the minicell generating strain after 4 h was continuing

to grow. It is noted in the combined mutant that DNA increases at a somewhat faster rate but cell mass increase persists about 1-1/2 h longer than DNA synthesis.

In both instances when the OD was plotted against CPM (H^3 in DNA) and when the OD was plotted against the CPM/OD ratio, a marked disparity can be observed between the patterns of the double mutant and the minicell producing mutant at 45 C commencing at an OD of 0.33 (the OD at which DNA synthesis decline in the double mutant is noted). At an OD of 0.9, 2.6×10^4 CPM and 3.2 CPM/OD differences are deduced. Laconically, the defective DNA⁻ initiation marker is responsible for impeding DNA replication and growth at 45 C in the double mutant, whereas at 30 C these processes appear to be parallel in the two strains. Clearly, the dissimilarities between replication and growth performances are contingent on the absence or presence of the DNA initiation mutation, the only known inequality between the strains.

Genome Location by Autoradiography

Autoradiographs prepared at the time the culture was halved and one portion shifted to 45 C portray 54.5% of the cell length being covered by DNA grain clusters but at 2 h a drastic divergence is observed: at 30 C, 60.2% of the cell length is covered with DNA but at 45 C only 8.6% coverage is detected. Furthermore, by 4 h at 45 C there is a preponderance of lysed cells, the ones remaining displaying 5.8% of their excessive length carapaced with DNA clusters and lacking discernible divisions; whereas at 30 C the cells are heavily labeled excluding polar regions and minicells. This study establishes that between 0 and

120 min at 45 C limited DNA grain clusters are not dispersed in the same fashion as at 30 C; instead the small grain clusters are separated by vast barren regions of growth whereas at 30 C the shorter cells have nearly continuous coverage of DNA barring the polar areas, thereby demonstrating once again the way in which the DNA⁻ initiation gene is affecting the minicell producing strain. Nuclear material is not detected at the extremities of "normal" cells, in short rods, or in minicells.

Growth and Division Studies

Growth and division studies were achieved by photographing spore outgrowths at 30 C and others were grown at 30 C for approximately 5 h then shifted to 45 C. An attempt to germinate spores at 45 C resulted in lysing after minimal outgrowth and prior to division which normally occurred 6 to 8 h following germination at 30 C.

Probability of a Minicell Division. The probability of a minicell division at 30 C was 0.44 whereas under shift conditions was 0.40, essentially similar and most likely ascribable to processes previously initiated at 30 C perpetuating at 45 C. However, the probability of a minicell division is slightly greater than that reported by Coyne and Mendelson (15) for CU 403 Div IV-B1 (0.33) which might be attributable to technique discrepancies or possibly the defective DNA marker is indirectly casual. Nevertheless, of importance is that after 2 to 2-1/2 h lysing is eminent at 45 C which is not at 30 C. Also, occasional contorted cells are observed which have swollen, lemon shaped bulges. This phenomenon has previously been detected in DNA mutants by Bazill

and Retief (6) and Mendelson and Gross (64). Inception time of lysing is in accord with the results of DNA replication kinetics and autoradiographic studies.

Both the frequency of minicell divisions and total divisions is greatest 7 to 8 h following germination or about 2 h after transfer to 45 C after which time commencement of lysis becomes apparent.

An endeavor to correlate the probability of a minicell division and the length of a clone in hopes of determining whether the frequency of minicell divisions and/or total divisions would be altered as a function of growth in the absence of DNA synthesis was unsuccessful; possibly the reason being that DNA synthesis proceeded (due to multifork replication and leakiness) for the time span observed after which lysis became conspicuous therefore preventing any opportunity for increased cell length after cessation of DNA synthesis.

Length of Clones Versus Time. There exists considerable variation in length of clones at the same hour. However, the average length 1 h after the shift to 45 C was 117 μm , 223 μm at 2 h, and at 3 h, 245 μm which was not greatly changed from that at 2 h reflecting the diminishment of DNA synthesis and growth and inception of lysis which happens about 2 to 2-1/2 h succeeding the shift.

Linear Regression Analysis. Derivations from linear regression reveal slopes of 20.8 and 19.79 at 30 C for the minicell producing strain and the double mutant respectively thereby implying they both produce about the same number of divisions per unit length. This is not unexpected since the combined mutant should function similarly to the

minicell generating strain at this temperature. However, at 45 C the slope for the combined mutant is 24.9 denoting division suppression probably imputable to impairment of the dnaB initiation marker. Significant and lacking is a slope for CU 403 Div IV-B1 at 45 C.

Percent Division Equivalents Expressed. Calculation of percent division equivalents expressed was determined for CU 403 Div IV-B1, 134ts grown at 30 C and 45 C based upon CU 403 at 30 C and 45 C and upon CU 403 Div IV-B1 at 30 C. Little difference is detected in the combined mutant at 30 C and 45 C for at both temperatures it is dividing about 1/4 and 1/2 as often as CU 403 at 30 C and 45 C, respectively, and almost 2/3 as frequently as CU 403 Div IV-B1 at 30 C. The double mutant appears less suppressed compared to CU 403 at 45 C rather than at 30 C presumably because fewer divisions would be expected at 45 C due to cell size at division increasing with the growth rate according to an exponential fashion (55).

Based upon CU 403 at 30 C, a 38.75% division equivalent expressed was determined for CU 403 Div IV-B1 (15) indicating the double mutant (24.1%) exhibits 15% greater suppression. Concisely, most likely attributable to malfunctioning of the dnaB marker is that the combined mutant is dividing less frequently than those strains to which it is compared and lysis begins approximately 2 to 2-1/2 hours following the shift.

Recapitulation

In order to assess the results of these studies a review of what is fundamentally taking place in the double mutant is offered:

1. DNA synthesis is diminished about 1-1/2 h after transfer with an increase of 8 fold and cell mass proceeds an additional 1-1/2 h with an increment of 8 fold; the minicell producing strain synthesized DNA 3-1/2 h after transfer with a 13 fold increase and cell mass is still climbing at 4 h.
2. There exists a marked discrepancy concerning the CPM (H^3 -DNA) versus OD and CPM/OD versus OD graphs in the behavior of the combined mutant and the minicell producing strain at 45 C beginning at an OD of 0.33 (the time of DNA cessation) with the double mutant leveling off and the minicell strain increasing.
3. Autoradiographic studies portray at 120 min subsequent to halving of the culture a disparity of 60.2% and 8.6% of the cell length covered with DNA grain clusters at 30 and 45 C respectively inferring reduction of DNA synthesis between 30 min and 1-1/2 h at 45 C. Additionally longer cells were observed at 45 C than at 30 C.
4. Average clonal length following the shift to 45 C is similar at 2 and 3 h suggesting retardation of DNA synthesis is exerting influence on growth.
5. Approximately 2 to 2.5 divisions are required to produce a minicell division at 30 C and 45 C. Both minicell divisions and total divisions occur 6 to 8 h succeeding germination after which lysis commences at 45 C. No correlation between the probability of a minicell division and cell length was noted.

6. Linear regression analysis and calculation of percent division equivalents indicates fewer divisions per clonal length are occurring than expected.

Seemingly the control of the impaired dnaB marker on the minicell producing strain is exercised at 45 C as suggested by differences resulting from DNA replication kinetics, autoradiographic studies, and linear regression analysis; however imperceptible distinctions at 30 and 45 C resulting from calculations of percent division equivalents expressed might be interpreted in terms of the values used in computation occurring primarily at lengths less than 250 μm after which lysis soon began thereby offering no occasion to observe authentic incongruities in growth and division patterns. More precisely, it is unfortunate that cell division and growth at the time of lysing continued to be under authority of DNA's instructions instigated at 30 C.

Conclusion

Certain vital questions, primarily what is responsible and by what process do abnormal divisions take place and how does cell division transpire in the absence of DNA synthesis, have confronted investigators for some time. In pursuit of credible and defensible explanations to these queries, this project was undertaken.

To implement this study a DNA initiation and minicell producing combined mutant was exploited. It was hoped deviations in division patterns at 45 C in the absence of DNA replication would be observed; for instance following reduction of DNA synthesis and allowing time for residual effects, a shift in the probability of minicell divisions might

have been noted but since this phenomenon never occurred due to reasons previously specified the initial questions remain unsolved and speculations only can be proposed.

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