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DNA Replication in Mesophilic and Thermophilic Bacteria

Pawan K. Gupta
Western Michigan University

DNA REPLICATION IN MESOPHILIC AND THERMOPHILIC BACTERIA

by

Pawan K. Gupta

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Pawan K. Gupta

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INTRODUCTION

The first isolation of thermophilic bacteria was done by Miquel (1). Thermophiles are bacteria which grow at much higher temperatures (55-80°C) than mesophiles which grow at more moderate temperatures (20-45°C).

Three major theories have been proposed to account for the phenomenon of thermophily. The first of these theories, developed by Gaughran (1), considers thermophily to be due to the presence of heat stable lipids. The second theory, proposed by Allen (2), views thermophily as a system characterized by rapid rates of synthesis and degradation. The third theory ascribes thermophily to physical-chemical differences between macromolecules of the thermophile and those of the mesophile.

The last theory has received most support thus far. The evidence comes largely from comparative studies of proteins and nucleic acids from thermophilic and mesophilic bacteria. Unusual thermal stability of cytoplasmic proteins from thermophilic organisms has been reported by Koffler et al. (3). The α-amylase isolated from Bacillus coagulans, grown at 55°C, showed greater heat stability than the same enzyme isolated from B. coagulans grown at 37°C (3). Glyceraldehyde 3-phosphate dehydrogenase (4,5), aspartokinase (6) and peptidase (7) from B. stearothermophilus showed a higher degree of thermal stability.

In our laboratory, Stenesh, Roe and Snyder (8) showed that de-

oxyribonucleic acid (DNA) from thermophilic strains of Bacillus was more heat stable and had a higher guanine plus cytosine (G+C) content than the DNA from the mesophilic strains of the same genus. Similar results were obtained in a study of the ribosomal ribonucleic acid (RNA) from the above strains of Bacillus (9). The ribosomes isolated from the thermophilic strains were also more heat stable than the ribosomes from the mesophilic strains (10). An in vitro protein-synthesizing system from a thermophile had a higher optimum temperature than a similar system from a mesophile (11,12). The lipid content of six strains of Bacillus has been studied and the thermophilic strains were shown to have a different fatty acid distribution than that of the mesophilic strains (13).

The replication of DNA by DNA polymerase has been shown to occur with a lack of fidelity (incorporation of non-complementary nucleotides) in in vitro systems from prokaryotes (14-16), eukaryotes (17,18) and viruses (19,20). There are suggestions that inaccuracies in DNA synthesis may be related to cellular aging (21), carcinogenesis (22), mutagenicity (23) and thermophily (24).

In our laboratory DNA and DNA polymerase have been isolated from the mesophile, B. licheniformis (growth range 37-45°C) and the thermophile, B. stearothermophilus (growth range 55-65°C) and the nature of the product of the DNA polymerase reaction has been investigated by means of nearest neighbor base frequency analysis (24,25). It was concluded that both DNA and DNA polymerase were involved in bringing

about a lack of fidelity during replication. Later studies in our laboratory measured the incorporation of deoxyribonucleoside triphosphates in the presence of synthetic polydeoxyribonucleotide templates (26). The results demonstrated again that there was a lack of fidelity in the in vitro replication catalyzed by DNA polymerase from these organisms. In view of these findings, it was of interest to determine the fidelity of DNA replication under in vivo conditions. This dissertation presents a study of the in vivo DNA replication during one cell cycle of synchronously growing cell cultures from two species of the genus Bacillus. One of these, B. licheniformis (B. lich), is a mesophile which was grown at 37°C and 45°C; the other, B. stearothermophilus (B. stearo), is a thermophile which was grown at 55°C and 65°C. As a measure of fidelity in DNA replication, the relative amounts of incorporation of the four bases were determined as a function of the growth temperature.

MATERIALS AND METHODS

Doubly distilled, deionized water was used throughout.

Biochemicals

Deoxythymidine (methyl-^{3}H, specific activity 81.2 Ci/mmole), deoxyadenosine (8-^{3}H, specific activity 14.45 Ci/mmole) and deoxycytidine (5-^{3}H, specific activity 24.7 Ci/mmole) were purchased from ICN Pharmaceuticals. Deoxyguanosine (8-^{3}H, specific activity 1.9 Ci/mmole) was purchased from Amersham Corporation. All labeled deoxyribonucleosides were found to be more than 99% pure as checked by thin layer chromatography. Cellulose powder (MN 300, gypsum free) was obtained from Brinkmann Instruments, Inc. Ribonuclease A (bovine pancrease, Type III A) and lysozyme were purchased from Sigma Chemical Corp.

Organisms and Growth Conditions

Cells of Bacillus licheniformis (NRS 243) and B. stearothermophilus 10 were grown in flasks containing 150 ml of nutrient liquid medium. The medium consisted of 1% Trypticase (BBL) and 0.2% Yeast Extract (Difco). Flasks containing the bacterial culture were incubated in an incubator shaker (New Brunswick Scientific Co., Model G25). Cells of B. licheniformis were grown at 37°C and 45°C and those of B. stearothermophilus were grown at 55°C and 65°C. Cell growth was followed by either absorbance measurements at 540 nm (Bausch and Lomb Spectronic 20) or by a viable cell count as described later.

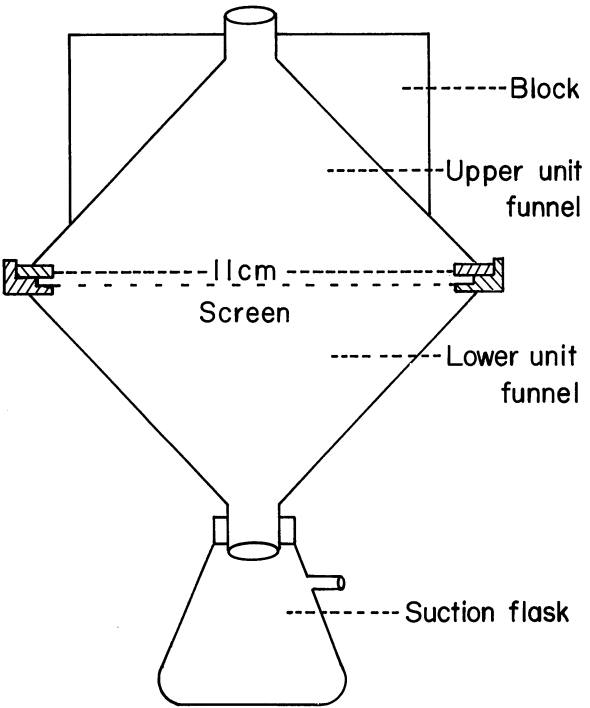
Synchronous Cultures

Synchronous growth was achieved by selecting and growing the smallest cells in a population. These cells were selected by filtering a culture under vacuum through a pad of Whatman no. 40 filter paper as described by Imanaka et al. (27) and Sargent (28). The entire procedure was as follows. Slants were first inoculated with cells from stock cultures and were incubated in a water bath overnight. The following morning, the cells were washed from two slants with 10 ml of sterile water and transferred to a flask containing 150 ml of sterile medium. Exponential growth was maintained for at least 12 generations and was not allowed to exceed the late log phase at any time. The late log phase corresponds to an absorbance (540 nm) of about 0.7 and 0.65 for the mesophile at 37°C and 45°C, respectively; it corresponds to about 0.65 and 0.60 for the thermophile at 55°C and 65°C, respectively. In order to achieve exponential growth for 12 generations the cells collected from the slants were transferred three times into fresh medium. At every inoculation enough cells (15-25 ml of liquid culture) were transferred into fresh medium (150 ml) to obtain an initial absorbance of about 0.1 at 540 nm.

The design of the filtration unit was similar to that described by Helmstetter (29). The internal diameter of the unit was 11 cm (Fig. 1). Two stainless steel, 500 ml funnels were used. The screen was a thin, 1 mm aluminum plate into which a large number of holes had been drilled. The top part of the unit was

FIGURE 1

Filteration apparatus



weighted down with a metal block to insure a tight seal even without clamps. For each 150 ml of the culture, the filtration unit was packed with six layers of Whatman no. 40 filter paper (11 cm) for the mesophile and with four layers for the thermophile. Three Whatman no. 40 washers were cut out to provide a tight seal around the edges of the screen. One washer was used above and below the pad and the third one was placed in the middle of the pad. The filter paper pad was then moistened with distilled water and tamped down on the lower part of the filtration unit. Distilled water, 100 ml, was then slowly passed through the pad under suction from a water aspirator. The suction flask and the funnel assembly were sterilized separately. The culture (after 12 generations of growth) was filtered through this unit in about one minute with suction from the water aspirator. One hundred and fifty ml of bacterial culture in midlog phase were filtered at a time. The midlog phase corresponds to an absorbance of 0.55 and 0.45 for the mesophile at 37°C and 45°C, respectively; it corresponds to an absorbance of about 0.50 and 0.45 for the thermophile at 55°C and 65°C, respectively. The filtrate, containing the smallest cells in the culture, was returned to the incubator. The entire filtration process took less than 2 minutes and was performed at room temperature except that the suction flask in the filtration unit was placed in a water bath and kept at the same temperature as that of the culture being filtered. During incubation of the filtered culture, 5 ml aliquots were removed at 5

or 10 min intervals for absorbance measurements. A portion of each aliquot was used for viable cell count.

Viable Cell Count

Petri dishes (15 cm diameter) were used to prepare nutrient agar plates containing 1% Trypticase, 0.2% Yeast Extract and 2% Bacto agar (Difco). Duplicate samples of cell culture were each diluted to about 6×10^3 cells/ml and two 0.05 ml of each dilution were plated on separate nutrient agar plates under sterile conditions. The plates were incubated at 37°C for the mesophile and at 55°C for the thermophile. After 24 hours the cell colonies were counted. Dilutions were such that there were not more than 400 colonies per plate. For each sample the average of the four plate counts was recorded.

DNA Biosynthesis

The cell culture isolated by filtration was immediately (prior to incubation) labeled with 10 μCi of a deoxyribonucleoside. In case of deoxythymidine, the labeled compound was added along with unlabeled deoxythymidine (6 μg, decreasing the specific activity of labeled deoxythymidine was necessary in order to obtain linear DNA biosynthesis for at least 60 min). Samples of 0.5 ml were removed from the culture at 15 to 20 min intervals and transferred to a test tube containing 5 ml of ice-cold 2M formic acid (30). After thoroughly mixing, the tube was kept in ice for 15 min. The mixture

was then filtered through a Millipore filter (25 mm diameter, Type HA, 0.45 μm pore size) followed by rinsing once with 5 ml of 2M formic acid and twice with 10 ml of saline buffer (1.5 mM in sodium citrate and 1.0 mM in sodium chloride). The Millipore filter was air dried, and placed in a scintillation vial together with 10 ml of Bray's scintillation fluid (100 gms PPO, 1.25 gms POPOP, 100 gms napthalene in 1 liter of p-dioxane; Nuclear Equipment Chemical Corp.) and counted in a liquid scintillation counter (Isocap/300, Searle Analytic Inc.). The counts were corrected for background counts.

Deoxyribonucleoside Pools

For determination of the intracellular level of a given deoxyribonucleoside (pool), the cell culture obtained after filtration was immediately labeled with either 10 or 50 μCi of the particular deoxyribonucleoside. Samples of 10 ml were withdrawn from the culture at 0, 1, 5, and 10 min intervals after the addition of isotope and transferred to a centrifuge tube kept in ice. The samples were quickly centrifuged at 120,000 x g for 10 min in a Sorvall centrifuge. The cell pellet was washed twice with 10 ml of saline buffer. Finally, 0.1 ml of 2M formic acid was added to the cell pellet and the mixture kept at 4°C for 15 min (31). The residue was removed by centrifugation at 120,000 x g for 10 min and the supernatant was used to determine the pool size of the free deoxyribonucleoside in the cells. An aliquot of the supernatant (5 μl) was spotted on glass fiber filter paper (Whatman GF/C, 2.4 cm diameter) and counted

to determine the total amount of radioactivity in the cell. An identical aliquot of supernatant was then applied to a thin layer chromatography (TLC) plate to separate the deoxyribonucleosides and the amount of radioactivity in the particular deoxyribonucleoside was then determined.

Thin Layer Chromatography

Glass plates (20x20 cm) were coated with 0.30 mm of cellulose powder (MN 300) using a TLC spreader (DESAGA, Brinkmann Inc.). Cellulose powder (11.25 gm), suspended in 80 ml of water and thoroughly mixed in a blender, was sufficient to coat three TLC plates. Chromatography was run in conventional TLC chambers (Brinkmann Inc.) using the following solvents: for separation of deoxyribonucleosides, 100 ml of solvent containing n-butanol and 0.6N ammonia in the ratio of 6:1 (v/v) (32); for separation of nucleic acid bases 100 ml of solvent containing n-propanol, hydrochloric acid (specific gravity 1.18) and water in the ratio of 65:17.2:17.8 (v/v) (33). When the TLC spots were to be counted, the cellulose powder was scraped off (spots were visualized with short wave ultraviolet light, Chromatovue, Ultraviolet Products Inc.) and transferred to a scintillation vial. Water (0.5 ml) was added and the mixture was thoroughly mixed on a vortex stirrer. The mixture was kept for one hour at room temperature before adding 10 ml of Bray's scintillation fluid. The vials were then left undisturbed until the solution cleared up (cellulose settled) before they were

counted. Essentially 100% of the cpm applied to the TLC plates were recovered using this procedure.

Isolation of DNA

The method described by Sarfert and Venner (34) was used except for the following modifications. After lysozyme treatment, sodium dodecyl sulfate was added to a final concentration of 1.5% and the mixture was incubated at 50°C for 10 min. Ribonuclease (150 µg/ml) treatment was done once overnight at 0°C. Traces of ether from the DNA solution, after the phenol extraction step, were removed by bubbling air (passed through a calcium chloride tube) through the solution while it was kept at 45°C in a water bath. The DNA was re-precipitated once and then dissolved in dilute saline citrate buffer (0.015M in sodium chloride and 0.0015M in trisodium citrate).

Analytical Determinations

All spectrophotometric measurements were made using a Zeiss PMQ-II Spectrophotometer.

Protein was determined by the method of Lowry et al. (35) using bovine serum albumin as a standard. Na_2CO_3 (50 ml of 2% solution in 0.1M NaOH) was mixed with 0.5 ml of 1% $CuSO_4.5H_2O$ and 0.5 ml of 2% Na-K tartrate. Three ml of this reagent was then mixed with 0.3 ml of the sample and allowed to stand at room temperature for 10 min. Folin Ciocalteu reagent (0.3 ml) was added with thorough mixing. After 10 min at room temperature, the absorbance was measured at 750 nm.

RNA was determined using the orcinol reaction (36). Three ml of 0.1% $FeCl_3$ in HCl was combined with 3 ml of the sample followed by the addition of 0.3 ml of the orcinol solution (100 mg/ml in 95% ethanol). The tubes were heated for 40 min in boiling water. After cooling to room temperature, the absorbance was measured at 670 nm.

DNA was determined using the diphenylamine reaction (37) with calf thymus DNA as a standard. Two ml of diphenylamine reagent (1 gm of diphenylamine was dissolved in 100 ml of glacial acetic acid and 2.75 ml of concentrated sulfuric acid were added) were combined with 1 ml of sample. The mixture was heated for 10 min in boiling water. After cooling to room temperature, the absorbance was measured at 595 nm. The absorbance of the DNA solution was also determined directly at 260 nm and quantitated using an extinction coefficient of 250 (1% solution, 1 cm light path) (38).

Hydrolysis of DNA

Isolated DNA solution was kept in small pyrex test tubes with screw caps. The solution was gently evaporated to dryness under vacuum in a desiccator, followed by the addition of 0.5 ml of 98% formic acid. The test tubes were capped tightly and heated in an oven at 110°C for one hour (39). After heating, the tubes were taken out and cooled to room temperature before opening the caps. The hydrolysate (1 µl) was spotted on TLC plate and the bases were separated and counted as described above.

Radioactive Methods

Disposable polyethylene gloves were used while handling radioactive materials. For labeling of the cell culture with radioactive deoxyribonucleosides, the required amount of isotope was first withdrawn from the stock solution with 10 µl Hamilton syringe and added to 5 ml of sterile water. The 5 ml were then poured (under sterile conditions) into the flask containing the culture. Disposable lambda pipettes (Drummond Scientific Co.; 1, 5, and 10 µl) were used for spotting radioactive solution on TLC plates and glass fiber paper and were found to be accurate within ±1%. After completion of experiments involving radioactive materials, glassware and scintillation vials were thoroughly washed and checked for residual radioactivity before being reused.

RESULTS AND DISCUSSION

PART I

Growth Curves

Typical growth curves of B. licheniformis at 37°C and 45°C and of B. stearothermophilus at 55°C and 65°C are shown in Fig. 2. Growth of the cells, as determined by absorbance measurements, leveled off at an absorbance of about 1.5 for both organisms. The doubling times, calculated from the logarithmic phase of growth are given in Table I.

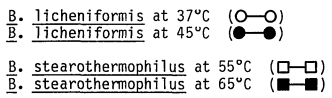
Both the mesophilic and the thermophilic bacteria grew at a faster rate at the higher temperatures. Care was taken so that in all of the subsequent experiments, the cultures were always grown under identical conditions, for example, speed of incubator shaker, time of overnight incubation of slants and volume of nutrient medium. It was observed that a slight variation in these standard conditions caused a change in the growth pattern of the organism.

Synchronous Cultures

Various methods were tried in an attempt to obtain synchronous cell division. Cultures of synchronously dividing bacteria can be obtained either by selecting and growing cells of a particular age class or by imposing synchrony. The latter may be achieved, for example, by amino acid starvation (40), by stationary phase treatment (41), or by density gradient centrifugation (42). These methods must distort temporal relationships of cell processes, although

FIGURE 2

Growth Curves



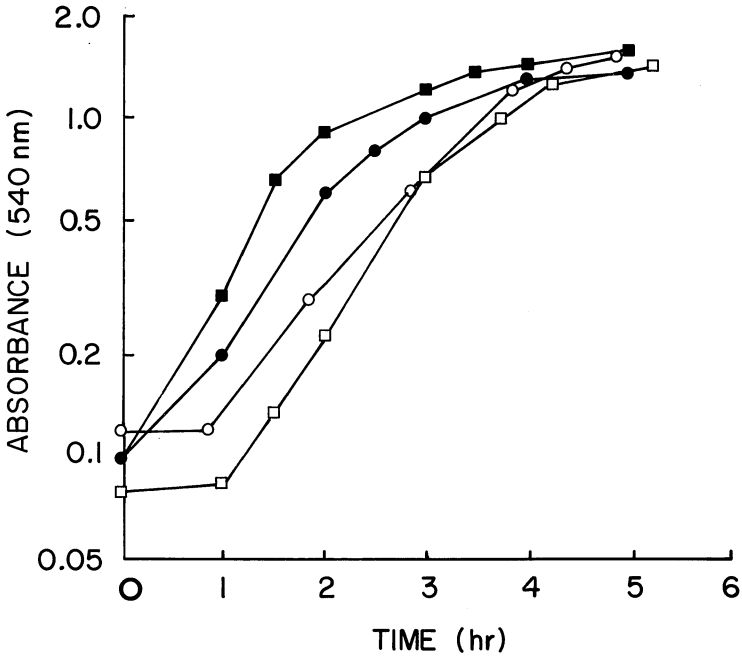


TABLE I

Growth Characteristics of *Bacillus*

Organism	Growth Temperature (°C)	Doubling Time (min)
B. licheniformis	37	51
	45	39
B. stearothermophilus	55	40
	65	34

damaging stresses may also be incurred with selection methods. The procedure followed here is a simple reproducible method for obtaining a synchronous culture based on size selection that causes minimum stress and gives sufficient yield for biochemical experiments.

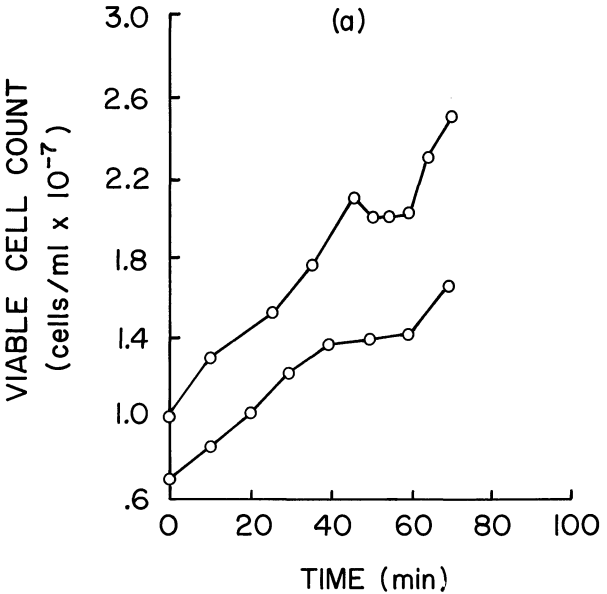
The filtration techniques of Imanaka et al. (28) was finally selected as the method yielding best results. Numerous filter papers and filter piles having different characteristics were tried. The most reproducible synchrony was obtained using six layers of Whatman no. 40 filter for the mesophile and 4 layers for the thermophile. The growth curves of the filtered (synchronous) cells are shown in Fig. 3 and 4. Two experiments were performed for each organism at a given temperature. In Tables II-V, the growth of these synchronous cells (one experiment) is given in terms of viable cell count and absorbance. Zero time refers to the end of the filtration process. In Table VI are listed the time intervals for one cell cycle (exact doubling of the number of viable cells obtained after filtration). These values were obtained from Figures 3 and 4. For example, for B. stearothermophilus at 55°C, the number of viable cells at zero time (time after filtration) was 3.8×10^6 and it doubled to 8.0×10^6 in 35 minutes and then remained constant until 50 min. Beyond that time, the number of cells increased again indicating the beginning of the second cell cycle. Therefore 50 minutes will be the time interval for one cell cycle for B. stearothermophilus cells at 55°C.

FIGURE 3

Growth curves of synchronous cultures

B. licheniformis (a) 37°C

(b) 45°C



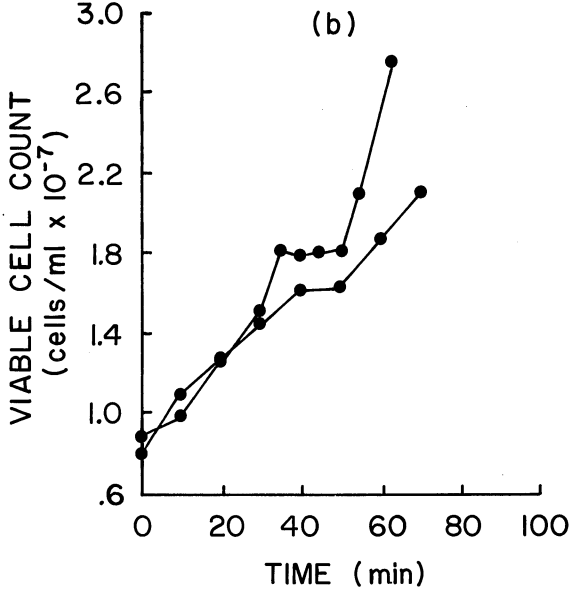
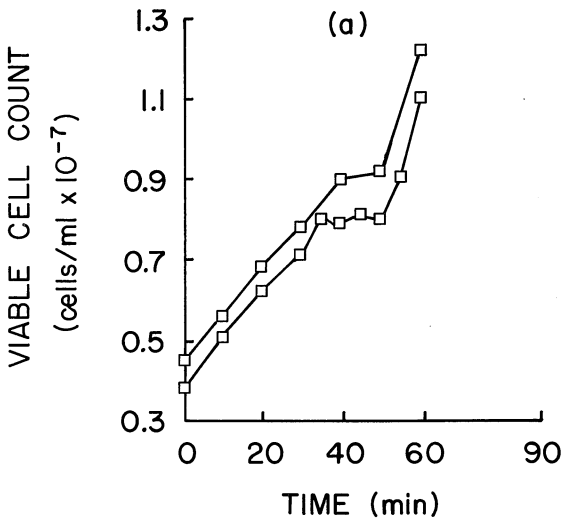


FIGURE 4

Growth curves of synchronous cultures

B. stearothermophilus (a) 55°C

(b) 65°C



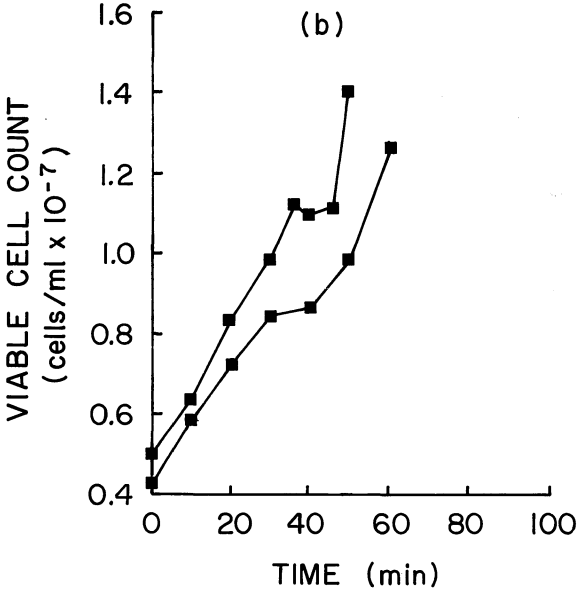


TABLE II

Growth of Synchronous Cultures

B. licheniformis-37°C

Time (min)	Absorbance (540 nm)	Viable Cell Count (cells/ml)x10^{-7}
0	0.100	1.02
13	0.120	1.35
26	0.135	1.56
36	0.155	1.78
46	0.170	2.12
51	0.185	1.93
56	0.195	1.98
61	0.210	2.02
66	0.230	2.32
71	0.260	2.51

TABLE III

Growth of Synchronous Cultures

B. licheniformis-45°C

Time (min)	Absorbance (540 nm)	Viable Cell Count (cells/ml)x10^{-7}
0	0.080	0.89
10	0.115	0.99
20	0.135	1.26
30	0.150	1.48
35	0.160	1.82
40	0.175	1.79
45	0.180	1.80
50	0.190	1.82
55	0.210	2.10
63	0.240	2.78

TABLE IV

Growth of Synchronous Cultures

B. stearothermophilus-55°C

Time (min)	Absorbance (540 nm)	Viable Cell Count (cells/ml)x10^{-7}
0	0.070	0.38
10	0.090	0.51
20	0.105	0.62
30	0.120	0.71
35	0.130	0.81
40	0.135	0.78
45	0.145	0.81
50	0.150	0.80
55	0.160	0.90
60	0.180	1.02

TABLE V

Growth of Synchronous Cultures

B. stearothermophilus-65°C

Time (min)	Absorbance (540 nm)	Viable Cell Count (cells/ml)x10^{-7}
0	0.080	0.49
10	0.100	0.63
20	0.120	0.83
30	0.140	0.98
36	0.155	1.12
41	0.165	1.09
46	0.170	1.11
51	0.200	1.41
56	0.220	1.82
61	0.240	2.32

Table VI

Synchronous Cultures of *Bacillus*

Organism	Growth Temperature (°C)	Length of Cell Cycle (min)
B. licheniformis	37	60
	45	50
B. stearothermophilus	55	50
	65	45

It was found in repeated experiments that the time interval for one cell cycle was reproducible to within 5 minutes or less. In fact, 5 min was also the time required to withdraw a sample from the culture, make dilutions, and plate the cells on the nutrient agar plates before the next sample could be withdrawn. While samples could have been withdrawn at time intervals of less than five minutes, this would have necessitated storing samples prior to analysis. This was undesirable since a sample of cell culture, stored at room temperature, tended to increase in viable cell count due to growth while it decreased in viable cell count due to lysis if stored at 4°C.

It was also found that the filtered cells of B. licheniformis and B. stearothermophilus grew synchronously for only one cell cycle. The number of synchronous cells obtained after filtration in these experiments represented approximately 5% of the original number of asynchronous cells for the mesophile and 8% for the thermophile.

PART II

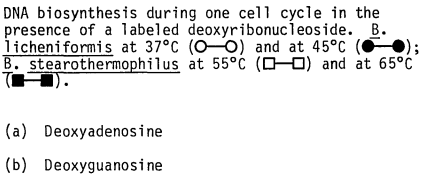
DNA Biosynthesis

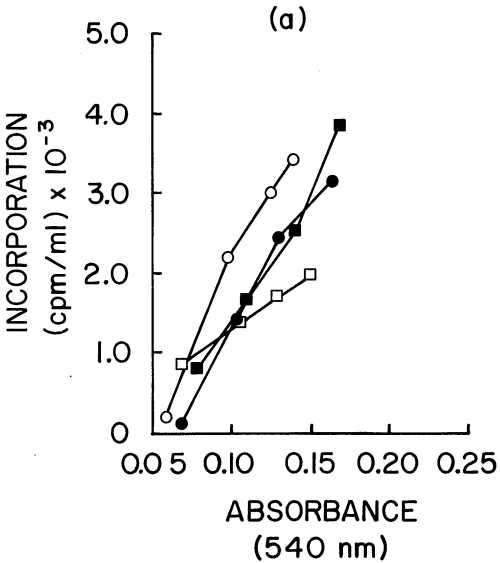
A given, labeled deoxyribonucleoside was added to synchronous cultures of B. licheniformis and B. stearothermophilus and the DNA biosynthesis was followed for one cell cycle. The results are shown in Fig. 5. It can be seen that DNA biosynthesis was continuous and approximately linear in both the mesophile and the thermophile for the duration of one cell cycle. This was true for all four deoxyribonucleosides. These metabolites are converted into their respective deoxyribonucleotides prior to being incorporated into the DNA (30). Further evidence for the incorporation of deoxyadenosine, deoxythymidine, deoxyguanosine and deoxycytidine into the DNA is described in Part III. It is shown there (by hydrolysis with 98% formic acid, followed by TLC) that in each case the isolated DNA (contaminated with less than 2% protein and less than 2.5% RNA) had essentially all of its label in that base with which it had been labeled; in other words, the metabolic interconversion of one nucleoside to another prior to incorporation into DNA was negligible.

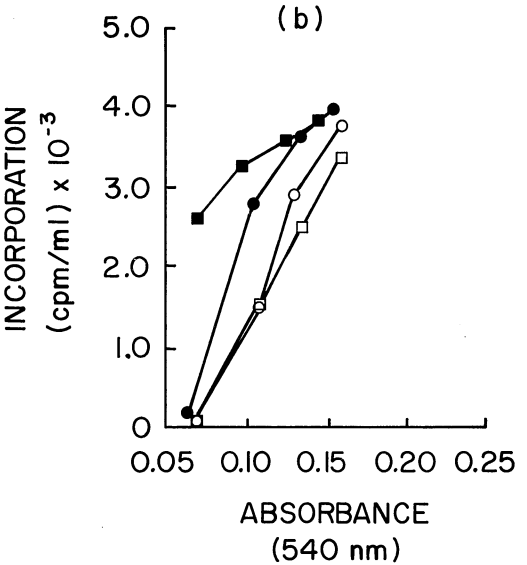
Deoxyribonucleoside Pools

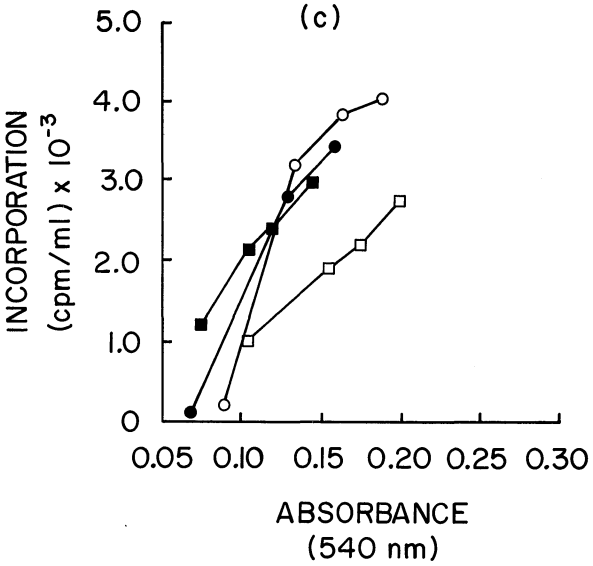
The experimental design was such that, after labeling of a cell culture with a given deoxyribonucleoside for one cell cycle, the DNA was isolated and the amount of label per unit of isolated DNA was determined. Any change in the amount of label per unit of isolated

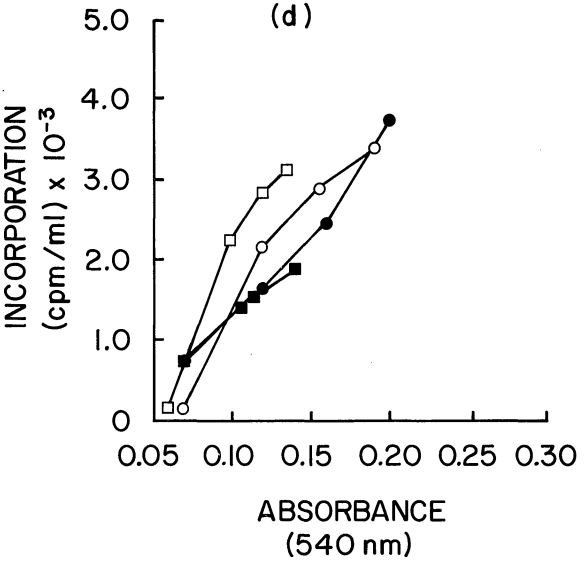
FIGURE 5











DNA (cpm/A_{260} unit) as a function of growth temperature of the cells would then be an indication of a lack of fidelity in DNA biosynthesis. For these conclusions to be meaningful, it was essential that an estimate be made of the total intracellular concentration of each deoxyribonucleoside at the start of the experiment. This was done in three different ways.

In one experiment (Table VII), 10 μCi of labeled deoxyribonucleoside was added to a synchronous culture. A 10 ml aliquot was removed immediately and kept at room temperature for 15 min. The cells were then collected by centrifugation and treated as described in Materials and Methods.

A second experiment (Table VIII) involved the addition of 50 μCi of labeled deoxyribonucleoside, removal of a 10 ml aliquot at time zero (immediately after addition of label), cooling it to 4°C, followed by immediate centrifugation and treatment as above.

A third experiment (Table IX) involved the addition of 50 μCi of labeled deoxyribonucleoside, removal of 10 ml aliquots at time 0, 1, 5 and 10 min, followed by centrifugation and treatment as above.

The first experiment permitted all cultures to reach a common temperature (room temperature) before the pool was determined. The second experiment attempted to prevent a change in the pool levels by cooling the aliquots to 4°C. The third experiment made an allowance for the possibility that establishment of a pool might be time dependent and vary with the organism and the growth temperature.

In all cases the intracellular concentration (pool size) of a labeled deoxyribonucleoside was calculated as follows:

$$\text{pool size (\%)} = \frac{\text{cpm of deoxyribonucleoside inside the cells}}{\text{total cpm inside the cells}} \times 100$$

The results of the three experiments are listed in Tables VII-IX. The variability between pool size values of different experiments as well as that between pool sizes of a given system as a function of time must be attributed to the fact that the counts in these experiments were very low, resulting in rather low reliability. In fact, some of the counts were so close to background levels that they have been omitted from Tables VIII and IX altogether.

Despite the low counts it is apparent from Tables VII-IX that the pool sizes in all cases have comparable values and the equilibrium values of these pools are attained within 1-5 min in all cases. What is most important, however, is the fact that all three experiments show that the pool of a given deoxyribonucleoside is the same for one organism at the two temperatures. In an attempt to obtain the total pool size for a given deoxyribonucleoside (that is labeled and unlabeled) larger samples of cultures (50 ml) were used. However, the concentration of unlabeled intracellular deoxyribonucleosides was so small that none could be detected by the TLC technique used. Deoxyribonucleosides are, in fact, not normal constituents of bacterial cells (30). Since approximately 0.1 µg of deoxyribonucleoside

TABLE VII

Intracellular Pool Size of Deoxyribonucleosides*

Organism	Deoxyribonucleoside			
	A	T	G	C
B. lich				
37°C	0.24	0.19	0.34	0.25
45°C	0.22	0.28	0.35	0.22
B. stearo				
55°	0.32	0.35	0.36	0.07
65°C	0.37	0.40	0.27	0.08

*Pool size in percentage
10 µCi of deoxyribonucleoside
15 min at room temperature

TABLE VIII

Intracellular Pool Size of Deoxyribonucleosides*

Organism	Deoxyribonucleoside			
	A	T	G	C
B. lich				
37°C	0.12	0.06	0.10	0.07
45°C	0.04	----	0.11	0.06
B. stearo				
55°C	0.08	0.10	----	0.03
55°C	0.07	0.06	0.18	0.09

*Pool size in percentage
50 µCi of deoxyribonucleoside
Immediate cooling to 4°C and centrifugation
No entry means that the counts were too low to be reliable (less than 20 cpm above background)

TABLE IX

Intracellular Pool Size of Deoxyribonucleosides*

Organism	Time (min)	Deoxyribonucleoside			
		A	T	G	C
B. lich					
37°C	0	0.05	0.07	0.04	0.05
	1	0.08	0.10	0.05	0.05
	5	0.09	0.18	0.06	0.05
	10	0.03	0.42	0.04	0.05
45°C	0	0.10	0.17	0.02	0.06
	1	0.07	0.17	0.05	0.07
	5	0.07	0.37	0.04	0.07
	10	0.04	0.26	0.06	0.07
B. stearo					
55°C	0	0.05	0.09	0.11	0.02
	1	0.06	0.07	0.04	0.01
	5	0.05	0.08	0.32	0.01
	10	0.02	0.12	0.11	0.01
65°C	0	0.06	0.07	0.11	0.01
	1	0.05	0.05	0.12	0.16
	5	0.03	----	0.12	----
	10	0.03	0.09	0.11	0.04

*Pool size in percentage
50 µCi of deoxyribonucleoside
Immediate cooling to 4°C and centrifugation
No entry means that the counts were too low to be reliable (less than 20 cpm above background)

are needed in order to be detectable on a TLC plate it can be estimated that, for deoxythymidine as an example, the unlabeled deoxyribonucleoside amounted to less than 4% of the labeled one added. The pool size of the labeled deoxyribonucleoside, as shown in Tables VII-IX, is therefore a very good approximation for the total intracellular pool size.

As mentioned above, the pool of a given deoxyribonucleoside is the same for one organism at two temperatures. It follows that any observed differences in the incorporation of a deoxyribonucleoside by a given bacterial system at two temperatures cannot be attributed to differences in the size of the initial deoxyribonucleoside pools. Consideration of deoxyribonucleoside pool size can, therefore, be omitted from an evaluation of the fidelity of DNA replication in these systems.

PART III

DNA Replication

DNA was isolated from B. licheniformis and B. stearothermophilus using the method of Sarfert and Venner (34). The DNA was found to contain less than 2% of protein and less than 2.5% of RNA. The amount of DNA was determined from the absorbance at 260 nm using an extinction coefficient of 250 (1% solution, 1cm light path) (38). The amount of isolated DNA, in terms of absorbance units at 260 nm, along with the total amount of radioactivity in that isolated DNA is listed in Table X. The data from the last column in Table X (cpm/A_{260} unit) are shown graphically in Figure 6. The two experiments did not yield identical cpm/A_{260} unit values due to the fact that neither the number of cells at the beginning of the experiment nor the amount of labeled deoxyribonucleoside added were exactly the same in the two experiments.

It should be noted that the cpm were not converted to dpm because the set of measurements for a given deoxyribonucleoside were all counted at essentially the same efficiency, making the conversion of cpm to dpm insignificant.

It can be seen from Table X and Figure 6 that, for B. licheniformis, there were significant differences in the incorporation of all four deoxyribonucleosides at 37°C compared to the incorporation at 45°C. On the other hand, the incorporation for B. stearothermophilus

TABLE X

DNA Replication

Organism	Amount of DNA (A_{260} units)		Label in DNA (cpm)		Cpm/A_{260} unit	
	a	b	a	b	a	b
B. lich 37°C						
A	13.42	90.98	5,240	900,910	390	9,912
T	16.83	90.85	60,588	433,144	3,600	4,765
G	37.39	86.18	330,587	1,224,639	8,841	14,210
C	53.04	76.23	504,134	1,333,780	9,504	17,496
B. lich 45°C						
A	30.50	40.39	147,789	729,402	4,845	18,052
T	23.58	57.23	114,166	397,252	4,841	6,941
G	53.54	109.60	207,207	884,885	3,870	8,073
C	72.24	54.59	711,711	1,179,337	9,852	21,600
B. stearo 55°C						
A	100.04	114.04	432,250	1,555,095	4,320	13,636
T	109.42	121.19	178,436	248,371	1,630	2,049
G	86.69	95.40	283,426	886,092	3,269	9,288
C	106.14	147.23	833,803	560,306	7,855	3,805
B. stearo 65°C						
A	56.34	148.33	258,031	1,939,506	4,500	13,075
T	124.56	97.63	62,318	142,813	500	1,462
G	156.31	148.30	540,519	1,428,047	3,458	9,629
C	112.18	65.94	964,748	282,240	8,600	4,280

a and b represent two separate experiments; the added labeled deoxyribonucleoside is indicated on the left.

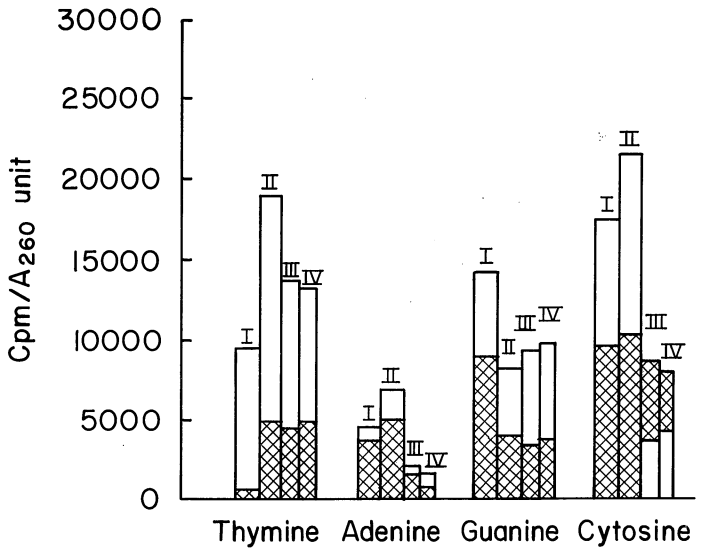
FIGURE 6

DNA replication

B. licheniformis 37°C (I)
45°C (II)

B. stearothermophilus 55°C (III)
65°C (IV)

Shaded area - experiment a
Unshaded area - experiment b



at 55°C was essentially identical to that at 65°C except for the case of deoxythymidine. The absolute differences in (cpm/A_{260} unit) as a function of temperature are listed in Table XI. A still better way of analyzing the data is shown in Table XII. Here the ratio of (cpm/A_{260} unit) values obtained for each organism at the two temperatures has been calculated. A ratio of one would indicate no change in the amount of incorporation at the higher temperature, while a ratio greater than one would mean decreased incorporation and a ratio less than one would indicate increased incorporation of a particular deoxyribonucleoside at the higher temperature. As can be seen from Table XII, there was a definite increase in the incorporation of adenine and thymine, a slight increase in the incorporation of cytosine, and a definite decrease in the incorporation of guanine for B. licheniformis as the growth temperature was increased from 37°C to 45°C. For B. stearothermophilus, on the other hand, there was a definite decrease in the incorporation of thymine but no significant changes in the incorporation of adenine, guanine and cytosine as the growth temperature was increased from 55°C to 65°C.

As mentioned, the isolated DNA was of high purity. Moreover, essentially all of the label in the isolated DNA was in that deoxyribonucleoside originally added in labeled form. This was true for both organisms, at both temperatures and for all four deoxyribonucleosides. These results are shown in Table XIII. These results were obtained by hydrolyzing the isolated DNA and separating the

TABLE XI

Changes in (cpm/A_{260} unit) with Growth Temperature

Organism	Base			
	A	T	G	C
B. lich				
37°C to 45°C				
(a)	+4455	+1247	-4971	+348
(b)	+8140	+2176	-6137	+4104
B. stearo				
55°C to 65°C				
(a)	+180	-1130	+180	+745
(b)	-550	-587	+341	+475

a and b represent two separate experiments

TABLE XII

Ratio of (cpm/A_{260} unit) for Two Growth Temperatures

Organism	Base			
	A	T	G	C
B. lich				
37°C/45°C (a)	0.08	0.74	2.28	0.96
(b)	0.55	0.68	1.76	0.81
B. stearo				
55°C/65°C (a)	0.96	3.26	0.94	0.91
(b)	1.04	1.40	0.96	0.89

a and b represent two separate experiments

TABLE XIII

Location of Added Label in Isolated DNA

Organism	Cpm			%*
	Hydrolysate	Ist TLC	IInd TLC	
B. lich 37°C				
A	19152	15325	3400	98.0
T	3022	2550	300	94.3
G	26034	27515	--	100.0
C	23326	21130	1700	98.0
B. lich 45°C				
A	15506	11725	3100	95.6
T	3425	2780	570	97.8
G	17961	11460	6150	98.0
C	20625	17850	2070	96.6
B. stearo 55°C				
A	33059	27500	4400	96.6
T	3781	3050	620	97.0
G	18837	17515	1050	98.5
C	9979	6090	3050	93.9
B. stearo 65°C				
A	41231	35485	4500	97.0
T	1320	1250	--	94.6
G	30359	27005	2625	97.6
C	4936	3780	900	94.6

* percentage of the label in the hydrolysate located in the two TLC spots.

four bases by TLC. It follows that metabolic interconversions of added deoxyribonucleosides were negligible so that results in Table XI represent real differences in deoxyribonucleoside incorporation.

The actual analysis of the DNA was carried out as follows. Five microliters of DNA hydrolysate were spotted on GF/C filter paper to determine the total amount of radioactivity in the hydrolysate. An identical aliquot was then spotted on a TLC plate, the four bases were separated, and the amount of radioactivity in the desired base was determined. Recovery in the appropriate base in one TLC run was incomplete due to tailing effects. Hence, the cellulose support from the remaining area of the TLC plate was collected and extracted. The extract was concentrated and respotted on a second TLC plate and the spot corresponding to the particular base was counted. The results of these experiments are shown in Table XIII.

The error rate of incorporation for each base was calculated as follows. Since the extinction coefficient of the DNA was taken as 250 (1% solution, 1 cm light path) it follows that one absorbance unit (A_{260}) of DNA represents 40 μg of DNA or 40 μg of total nucleotides. Using 250 as the average molecular weight of a nucleotide, it follows that 40 μg of total bases correspond to 1.6×10^{-4} mmole of total nucleotides.

Knowing the specific activity for each deoxyribonucleoside (see

Materials and Methods) the observed differences in cpm per A_{260} unit (Table XI) could then be converted into changes in mmoles of a given base per 1.6×10^{-4} mmole of total bases incorporated and hence the error rates can be calculated. These are listed in Table XIV. Thus, for example, for B. licheniformis (Experiment a), there is an erroneous incorporation of one extra adenine for every 3.0×10^{5} total bases incorporated as the temperature is raised from 37°C to 45°C. For guanine in the same experiment, however, the results indicate that at the higher temperature there is one less guanine incorporated for every 3.7×10^{4} total bases incorporated. In these calculations it was assumed that the specific activity of the added deoxyribonucleoside does not change inside the cells. This is a fair assumption since, as mentioned earlier, deoxyribonucleosides are not the normal constituents of bacterial cells and in fact, could not be detected by the chromatographic techniques used in this study. It is further assumed that the specific activity of the added deoxyribonucleoside is identical to that of the deoxyribonucleotide which is the final substrate for the enzymatic incorporation in the DNA.

Two conclusions can be drawn from the results shown in Table XIV. First, generally speaking, there is greater lack of fidelity in the replication of DNA in the mesophile than in the thermophile. It is tempting to speculate that it is precisely because of an ability to carry out DNA replication with an unusually high degree of fidelity that thermophiles can survive at elevated temperatures.

TABLE XIV

Error Rates of Deoxyribonucleoside Incorporation

Organism		Base			
		A	T	G	C
B. lich 37°C to 45°C	(a)	$1/3.0 \times 10^5$	$1/3.1 \times 10^4$	$1/3.7 \times 10^4$	$1/6.6 \times 10^6$
	(b)	$1/1.7 \times 10^5$	$1/1.8 \times 10^4$	$1/2.9 \times 10^4$	$1/5.7 \times 10^5$
B. stearo 55°C to 65°C	(a)	$1/7.7 \times 10^6$	$1/3.4 \times 10^4$	$1/1.0 \times 10^6$	$1/3.1 \times 10^6$
	(b)	$1/2.5 \times 10^6$	$1/6.6 \times 10^4$	$1/5.3 \times 10^5$	$1/5.0 \times 10^6$

a and b represent two separate experiments

Second, the error rates for the in vivo replication of DNA are decidedly smaller than those reported for in vitro systems by Battula and Loeb (43) and by Stenesh and McGowan (26). This is of course expected in order for the organism to be able to transmit genetic information from generation to generation with a high degree of fidelity.

It must be stressed that the error rates computed here need not necessarily represent actual misincorporation of bases in the DNA. Conceivably, temperature-dependent changes in certain metabolic activities (for example, conversion of deoxyribonucleosides to deoxyribonucleotides, hydrolysis of deoxyribonucleotides, changes in pool size, and changes in transport across the cell membrane) could affect the pool of labeled deoxyribonucleotide available and hence the amounts of label incorporated into the DNA. Yet even if this were the case, the data definitely show significant differences in the DNA replicating mechanisms of each organism at two temperatures as well as differences between the organisms.

Moreover, a number of experimental observations indicate that the error rates do indeed represent actual misincorporation of bases rather than metabolic changes. These observations include the following. There is no consistent trend for the error rates either for the four bases and one organism or for a given base and the two organisms. The level of error rates is reasonable and significantly less than that found for in vitro experiments. The ratio of cpm/A_{260}

unit (Table XII) for two growth temperatures is essentially one for some cases and definitely different from one (either greater or less than one) for others. The error rates are different for the mesophile and the thermophile. The pool sizes reach their equilibrium values within a very short time and then are maintained at a constant level. In many cases identical pool sizes resulted in quite different error rates. In fact, there is no consistent correlation between the pool size and the magnitude of the error rate. For example, the pool sizes at two temperatures are generally essentially identical, yet error rates are found and they were not identical. Or, considering B. licheniformis at 37°C for example, the pool sizes for all four bases were essentially identical, yet the error rates were not.

The amount of plasmid DNA found in Escherchia coli and Salmonella species (44) is less than 1% of the total DNA content in the cell. In the Bacillus species the amount of plasmid DNA present is likely to be of similar if not identical magnitude. If the plasmid DNA had replicated with complete lack of fidelity, the observed values of misincorporation would hardly be affected since in those cases where changes in incorporation were observed, these accounted to changes of the order of 50-100 %.

SUMMARY

DNA replication was studied in the mesophile, Bacillus licheniformis and in the thermophile, B. stearothermophilus. Cells were grown synchronously at 37°C and 45°C for the mesophile, and at 55°C and 65°C for the thermophile. The cells were labeled with a deoxyribonucleoside for one cell cycle and the quantitative incorporation of the deoxyribonucleoside into the replicating DNA was determined. It was found that, as the growth temperature was increased for the mesophile, there was a definite increase in the incorporation of adenine and thymine, a slight increase in the incorporation of cytosine and a definite decrease in the incorporation of guanine. For the thermophile, on the other hand, there was a definite decrease in the incorporation of thymine but no significant changes in the incorporation of adenine, guanine and cytosine as the growth temperature was increased. Calculated error rates of the incorporation of these bases indicate that DNA replication in the thermophile occurred with a greater fidelity than that in the mesophile. For both organisms, the in vivo error rates of incorporation were significantly lower than those reported for in vitro studies of DNA replication.

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