TEMPERATURE DEPENDENT PERMEABILITY CHANGES OF BACILLUS SUBTILIS AND INCORPORATION OF NUCLEOTIDES INTO DNA

R. C. HENNEBERRY and ERNST FREESE

Laboratory of Molecular Biology
National Institute of Neurological Diseases and Stroke
National Institutes of Health
Bethesda, Maryland 20014

Received September 24,1973

SUMMARY

Cells of B. subtilis exposed to temperatures between 0 and 5 C are permeable to small molecules not normally able to pass through the cell envelope. As a result, deoxyribonucleotide triphosphates (dXTPs) are incorporated into DNA if the reaction mixture contains all four dXTPs. Since this incorporation is insensitive to 6-(p-hydroxyphenylazo)-uracil and is not observed in DNA Polymerase I mutants, we conclude it reflects DNA repair rather than the DNA replication which can be observed in cells permeabilized with toluene.

INTRODUCTION

The sudden chilling of a bacterial suspension to 0 C ("cold shock") causes the release of UV absorbing material and loss of viability in a variety of microorganisms (5). Although cold shock was generally thought to have no effect on Gram positive bacteria (5), Smeaton and Elliott (11) reported that it caused the slow release of a ribonuclease inhibitor of Bacillus subtilis. We report here that cold treatment of B. subtilis causes the leakage of cell material and permits the incorporation of deoxyribonucleotide triphosphates (dXTPs) into DNA. These permeability changes occur at temperatures between 0 and 5 C, whereas treatment above 8 C is ineffective. Mutant and inhibitor studies

Copyright © 1973 by Academic Press, Inc.
All rights of reproduction in any form reserved.

show that the dXTP incorporation reflects DNA repair rather than the DNA replication observed in cells permeabilized with toluene (7).

MATERIALS AND METHODS

The strains of B. subtilis employed and their characteristics are shown in Table 1. Cells were grown in phosphate-

TABLE I

B. subtilis Strains

Strain	Source	Growth requirements	Relevant characteristics
60015	E. Nester (SB26)	trp, met	Standard strain
60437	B. Strauss (JB1-49-40)	trp, thy	Sensitive to UV and MMS
60439	B. Strauss (JB1-49-14)	trp, thy	Sensitive to UV but not MMS
61476	N. Brown (NB841)	trp, thy	DNA Polymerase I deficient
61304	J. Gross (ts134)	trp, thy	ts DNA B (initiation)

buffered nutrient sporulation medium (NSMP, ref. 6) supplemented with met (10 µg/ml), thy (5 µg/ml), and trp (25 µg/ml) where needed at 37 C in a reciprocating waterbath shaker to an absorbance at 600 nm (A_{600}) of 1.0. They were centrifuged at room temperature and washed with an equal volume of room temperature "phosphate buffer" (0.1 M K_2HPO_4-NaH_2PO_4, pH 7.4). Cells were normally cold shocked by suspending them in ice-cold phosphate buffer and incubating the suspension for 5 minutes on ice. When temperatures other than 0 C were used the phosphate buffer was equilibrated at the test temperature and the cells were incubated at this test temperature. To measure the release of UV absorbing material the cell suspension was filtered through a membrane filter (0.45 µ pore size, Millipore) and the A_{260} and A_{280} of the filtrate was determined. For measurement of [^{3}H]TTP incorporation, cells treated for 5 minutes at 0 C were transferred to 37 C and incubated 15 minutes; 6 x 10^8 of these cells were added to a reaction mixture (600 µliter final volume) containing K-PO_4 (70 mM, pH 7.4); $MgSO_4$ (1.3 mM); ATP (2 mM); and dATP, dCTP, dGTP (each 33 µM) and [^{3}H]TTP (33 µM, 0.25 µCi/nmole, New England Nuclear). 100 µliter samples were added to 2 ml ice-cold trichloroacetic acid (TCA, 10%) containing 0.1 M Na_4-pyrophosphate. After 15 minutes or more on ice, precipitates were collected on membrane filters that had been soaked in 5% TCA, washed twice with 15 ml cold 5% TCA, dried and counted in a Packard Tri-Carb spectrometer in 10 ml toluene containing RPI Scintillator (Research Products International).

RESULTS AND DISCUSSION

Cells of B. subtilis cold shocked at 0 C released about 4 times more UV absorbing material than cells treated in the same manner at room temperature (Table 2). The released material absorbed maximally at 260 nm, had an A_{260}/A_{280} ratio of about 3, and showed no hyperchromicity after heating to 100 C for 10 minutes. We have not attempted further characterization of this material but assume it consists of low molecular weight metabolites. Figure 1 shows that the release of UV absorbing material was greatest when cells were exposed to temperatures between 0 and 5 C and decreased at higher temperatures. Possibly, this phenomenon relates to the thermotropic phase transition temperature of the membrane lipids (4).

When the cold shocked cells were incubated for 15 minutes at 37 C and then added to a reaction mixture containing Mg^{++}, ATP, and the four dXTPs, they incorporated [^{3}H]TTP (TTP) into TCA precipitable material (Figure 2). Without cold shock, incorporation of TTP was poor. TTP is a direct precursor of DNA but cannot enter vegetative cells (10); cold shocking of B. subtilis apparently permeabilizes the cells to small molecules that normally cannot pass through the cell envelope.

The effects of various additions and/or omissions on incorporation of TTP in the complete reaction mixture are shown in Table 3. Thymine, thymidine, TMP, and TDP failed to dilute the TTP isotope, indicating that the nucleotide triphosphate itself rather than some breakdown product is incorporated. TTP incorporation was stimulated by ATP, which could not be replaced by adenosine, AMP, or ADP; CTP was somewhat stimulatory (Table 3).

The TTP incorporation assay used in these studies measures

TABLE 2

Release of UV absorbing material from cold shocked cells of B. subtilis*

Experiment No.	A_{600} of Culture	Absorbance of Filtrates 260 nm			280 nm		
		0 C	23 C	Ratio (OC/23C)	0 C	23 C	Ratio (OC/23C)
1	1.050	2.230	0.519	4.3	0.745	0.150	5.0
2	0.935	1.974	0.522	3.8	0.657	0.168	3.9
3	1.088	2.438	0.704	3.5	0.970	0.213	4.5

*Cells from 50 ml culture of B. subtilis strain 60015 were centrifuged and washed at room temperature. The pellet was suspended in 2 ml phosphate buffer at either 0 C or 23 C and after 5 minutes cell-free filtrates were prepared as described in Materials and Methods.

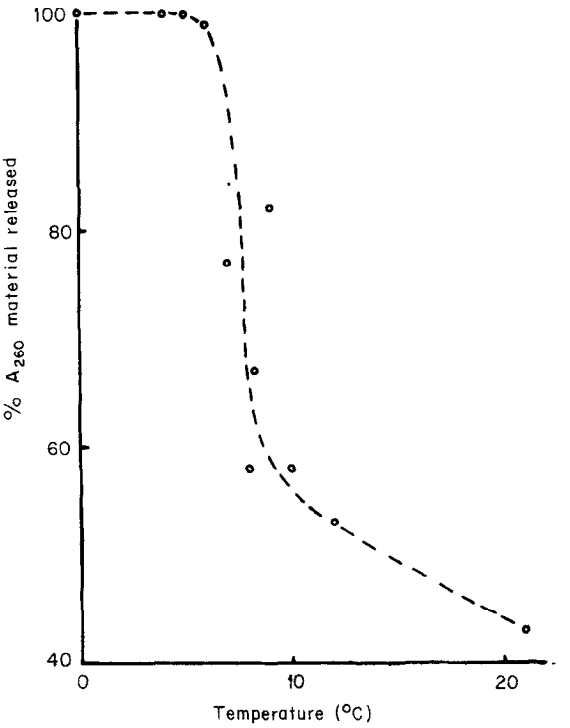


Figure 1. Absorbance (A_{260}) of cell-free filtrates from cells of B. subtilis strain 60015 cold shocked at the indicated temperature (see Materials and Methods).

extracellular as well as intracellular DNA polymerase activity. However, 80% of the incorporated TTP pelleted with the cells on low speed centrifugation (3,000 x g, 8 minutes); less than 1% of the label in this pellet was rendered TCA-soluble by pancreatic DNase (200 µg/ml, 20 minutes, 37 C). After treatment of the pellet with lysozyme (100 µg/ml, 30 minutes, 37 C) and DNase, 88% of the tritium became TCA-soluble. These results indicate that most of the TTP incorporation in cold shocked cells reflects intracellular DNA synthesis.

When a temperature sensitive DNA initiation mutant (61304, ref. 8) was shifted to 45 C for 45 minutes prior to cold shock, and TTP incorporation was then measured at 45 C, the same amount

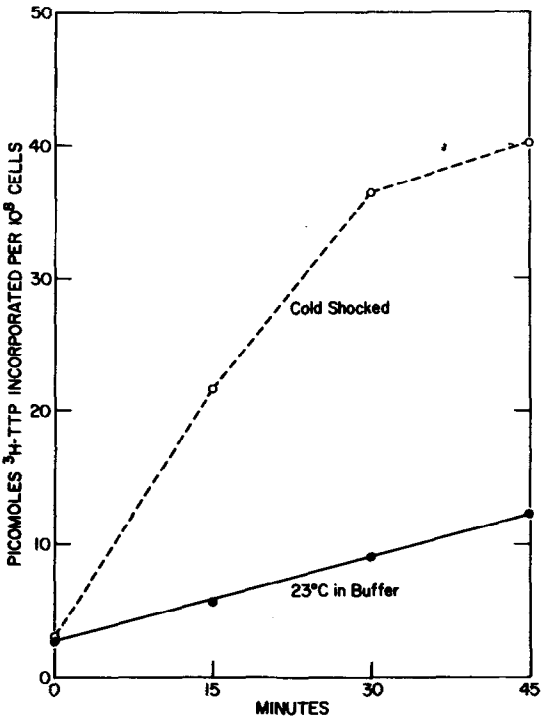


Figure 2. [^{3}H]TTP incorporated into TCA precipitable material by B. subtilis strain 60015 (see Materials and Methods).

---

of incorporation was observed as in the standard strain (Figure 3). Figure 3 again shows the stimulation of the reaction by ATP. Moreover, TTP incorporation in 61304 was insensitive to reduced 6-(p-hydroxyphenylazo)-uracil (HPUra, Figure 3), which is a specific inhibitor of DNA replication in B. subtilis (9). These results suggested that TTP incorporation in cold shocked cells reflected DNA repair rather than replication synthesis. To verify this conclusion, TTP incorporation was examined in cold shocked cells of several repair deficient mutants (Figure 4). Strain 60439, sensitive to UV but not to methyl methane sulfonate (MMS), incorporated TTP at least as well as the standard strain. In contrast, strain 60437, which is sensitive to both UV and MMS,

TABLE 3

Contents of Reaction Mixture	% [^{3}H]TTP Incorporated
Complete	100
" + thymine*	107
" + thymidine*	110
" + TMP*	83
" + TDP*	80
" + TTP*	6
" - $MgSO_4$	11
" - ATP	15
" - ATP + adenosine/AMP/ADP**	17-33
" - ATP + CTP**	55

*165 μM **2 mM

incorporated lower levels of TTP. The strain 61477, which has only 10% of the Polymerase I activity of the parental strain (B. Strauss, personal communication) incorporated TTP very poorly (data not shown). The mutant 61476, which has less than 0.5% of the normal Polymerase I activity (9), incorporated even less TTP (Figure 4). All these strains were permeabilized by cold shock as well as the standard strain, based on the comparison of UV absorbing material released.

We conclude that in cold shocked cells of B. subtilis TTP incorporation reflects DNA repair rather than replication. This conclusion may explain the necessity for the 15 minute incubation at 37 C before the addition of cold shocked cells to the TTP incorporation mixture. Incubation at 0 C or in the presence of

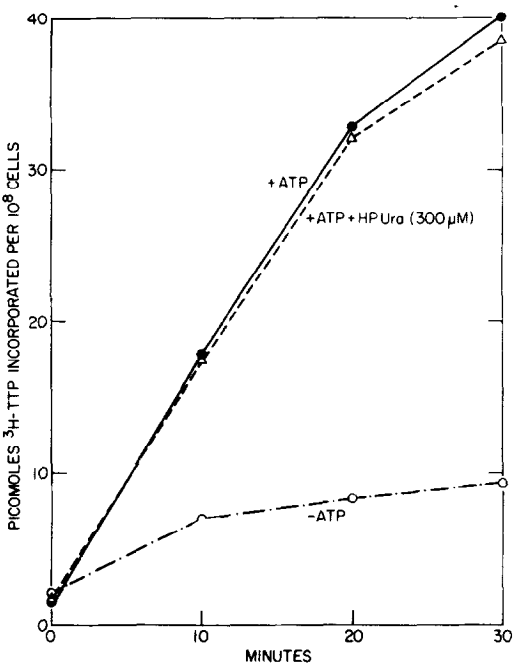
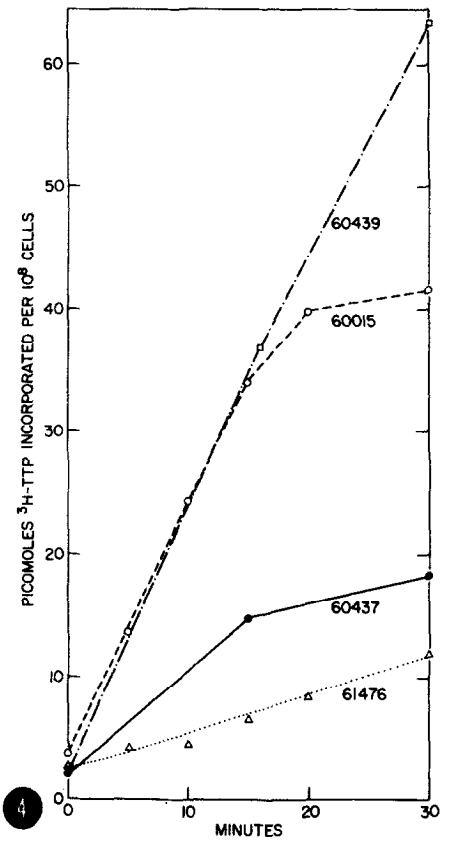


Figure 3. [^{3}H]TTP incorporated into TCA precipitable material by cold shocked (0 C) cells of B. subtilis strain 61304 (see Materials and Methods).

p-hydroxymercuribenzoate at 37 C reduced the subsequent TTP incorporation (Figure 5). Presumably, nuclease-mediated damage to DNA is a prerequisite for subsequent repair synthesis. Induction of repair by activation of endogenous nucleases has previously been reported in E. coli permeabilized with Tris-EDTA (1,2). An ATP-dependent deoxyribonuclease in B. subtilis has recently been reported (3). This enzyme is also inhibited by high ATP concentrations, which could explain the stimulation of TTP incorporation by ATP.

In contrast to cold shocking, permeabilization of the two repair deficient mutants (60437 and 61476) with toluene (7) allowed incorporation of TTP, which was sensitive to HPUra



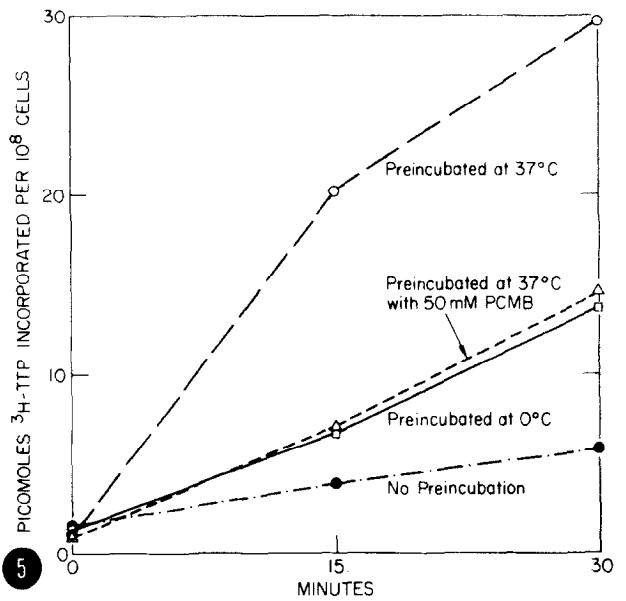


Figure 4. [^{3}H]TTP incorporated into TCA precipitable material by cold shocked (0 C) cells of the indicated strain of B. subtilis (see Materials and Methods).

Figure 5. [^{3}H]TTP incorporated into TCA precipitable material by cold shocked (0 C) cells of B. subtilis strain 60015 (see Materials and Methods).

---

and therefore represented DNA replication. However, cells which had been cold shocked before the toluene treatment incorporated about 50% less TTP than cells which received only the toluene treatment. Possibly, only about 50% of the cells were affected by the cold shock and the other 50% permeabilized by toluene. This could imply that permeabilization by cold shock affects a fraction (50%) of the cells in such a way that they can no longer replicate their DNA.

It is apparent that permeabilization of B. subtilis by exposure to low temperatures, as may inadvertently occur in a refrigerated centrifuge, can affect several cellular processes; this should be considered in the design of experiments.

REFERENCES

1. Buttin, G., and Kornberg, A. (1966) J. Biol. Chem. 241, 5419-5427.
2. Buttin, G., and Wright, M. (1968) Cold Spring Harbor Symp. 33, 259-269.
3. Chestukhin, A. V., Shemykin, M. F., Kalinina, N. A., and Prozorov, A. A. (1972) FEBS Letters 24, 121-125.
4. Esfahani, M., Limbrick, A. R., Knutton, S., Oka, T., and Wakil, J. (1971) Proc. Nat. Acad. Sci. USA 68, 3180-3184.
5. Farrell, J., and Rose, A. H. (1967) in Thermobiology, A. H. Rose, ed., pp. 147-218, Academic Press, New York.
6. Fortnagel, P., and Freese, E. (1968) J. Bacteriol. 95, 1431-1438.
7. Matsushita, T., White, K. P., and Sueoka, N. (1971) Nature New Biol. 232, 111-114.
8. Mendelson, N. H., and Gross, J. D. (1967) J. Bacteriol. 94, 1603-1608.
9. Neville, M., and Brown, N. C. (1972) Nature New Biol. 240, 80-82.
10. Rhinehart, K. V., and Copeland, J. C. (1973) Biochim. Biophys. Acta 294, 1-7.
11. Smeaton, J. R., and Elliott, W. H. (1967) Biochem. Biophys. Res. Commun. 26, 75-81.