INVOLVEMENT OF RNA POLYMERASE IN THE SYNTHESIS OF DNA BY GROWING AND TOLUENE-TREATED CELLS OF BACILLUS BREVIS*

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Summary: The incorporation of [^{3}H] thymidine into DNA by growing cultures of Bacillus brevis as well as other Bacilli was strongly inhibited by rifampicin and streptolydigin, suggesting a role for RNA polymerase in DNA replication. On the other hand, DNA synthesis in toluene-treated cells of B. brevis, which was dependent on the presence of ATP or other ribonucleoside triphosphates, was unaffected by streptolydigin or rifampicin. However, upon storage of toluene-treated cells at -20°, sensitivity to inhibition by streptolydigin developed progressively (up to 76% inhibition) over a period of 4 weeks, while the overall rate of DNA synthesis remained constant.

Considerable evidence has accumulated over the past several years that the initiation of DNA chains during replication involves an RNA primer (1-13). Nevertheless, the mechanism by which the priming RNA segments are synthesized is understood in only a few systems. The conversion of bacteriophage M13 DNA to its replicative form is inhibited by rifampicin (6, 7), suggesting an involvement of RNA polymerase, and this has been confirmed by experiments in a cell-free system (8). On the other hand, the replication of the closely related bacteriophage ϕX_{174} is not affected by inhibitors of RNA polymerase (8, 9) and must proceed by a different mechanism. The same applies to the replication of the E. coli chromosome, which, like ϕX_{174} and unlike M13, requires the products of genes dnaB, dnaC(D), and dnaG (14). Thus, if the replication of the bacterial chromosome and of ϕX_{174} requires RNA primers, these are not synthesized by the conventional RNA polymerase but must be produced by an unknown mechanism.

Since the mechanism of replication of the bacterial chromosome is of particular interest, I have turned to the study of DNA synthesis in Bacillus

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species in the hope that DNA replication in these organisms may be more amenable to investigation. Indeed, my results suggest that DNA replication in Bacilli may proceed by a simpler mechanism than in E. coli in that it involves the conventional RNA polymerase. In this paper, I describe the inhibition of DNA synthesis in growing Bacilli by rifampicin and streptolydigin as well as conditions under which DNA synthesis in toluene-treated cells of Bacillus brevis can be made sensitive to inhibitors of RNA polymerase.

MATERIALS AND METHODS

Materials. [2-^{14}C] uracil (7 Ci/mole), [methyl-^{3}H] thymidine (2 Ci/mmole) and [methyl-^{3}H] thymidine 5'-triphosphate (100 Ci/mole) were obtained from New England Nuclear. Other nucleotides, rifampicin and actinomycin D were purchased from Calbiochem. Streptolydigin was the gift of Dr. G. B. Whitfield of the Upjohn Company.

Bacterial Strains and Growth Conditions. Bacteria were cultivated on a rotary shaker at 37°. Bacillus brevis ATCC 8185 and B. subtilis ATCC 6051 were grown in a rich medium (15) and B. polymyxa ATCC 20591 and B. licheniformis ATCC 10716 in the synthetic medium of Biswas et al. (16). Bacillus brevis R15, a spontaneous rifampicin-resistant mutant, was selected from B. brevis ATCC 8185 by its ability to grow on Penassay agar containing 5 mg/ml of rifampicin. RNA synthesis in toluene-treated cells of this strain was not inhibited by rifampicin at a concentration (1 μg/ml) which strongly inhibited (90%) RNA synthesis in B. brevis ATCC 8185, showing that resistance was not due to a permeability change.

Pulse-Labeling Experiments. Samples of exponentially growing bacterial cultures (100-140 Klett units) were treated for 10 minutes with antibiotics as indicated. Samples (1 ml) (including a control without antibiotic) were then incubated for 1 minute with 0.1 μCi of [^{14}C] uracil, or with 1 μCi of [^{3}H] thymidine. The incorporation of the radioactive precursors into RNA or DNA was measured as alkali-sensitive or alkali-resistant radioactivity, respectively, insoluble in trichloroacetic acid, as described previously (17).

Toluene-Treatment of Cells. Permeable cells were prepared by treatment with toluene by a modification of the procedure of Matsushita et al. (18), as described previously (19). In some experiments, cells were incubated under conditions of DNA synthesis after toluene treatment. In that case, treatment with toluene was carried out in 0.1 M potassium phosphate buffer, pH 7.5, the cells were collected by centrifugation at 5,000 x g for 5 min at 4° and resuspended in 0.1 ml of this buffer containing also 10 mM $MgCl_2$, 1.5 mM ATP, 33 μM each of dCTP, dGTP, and dATP, and 20 μM dTTP. After incubation for 30 min at 37°, the mixture was diluted with 2 ml of potassium phosphate, pH 7.5, and centrifuged at 5,000 x g for 5 min. The resulting pellet was resuspended in 2 ml of potassium phosphate, pH 7.5, centrifuged again, and finally resuspended in 0.05 M triethanolamine hydrochloride, pH 7.5, - 50% glycerol and stored at -20°.

Assay of DNA Synthesis. DNA synthesis in toluene-treated cells was measured by the incorporation of [^{3}H] dTTP into acid-insoluble material as described by Moses and Richardson (20). The incubation mixtures (0.1 ml) contained 0.05 M triethanolamine hydrochloride, pH 7.5, 10 mM $MgCl_2$, 33 μM each of dCTP, dGTP, and dATP, 20 μM [^{3}H] dTTP, 1.5 mM ATP and 3-6 x 10^7 cells. Incubation was carried out at 37° for 30 min unless stated otherwise.

TABLE I

Effect of antibiotics on DNA and RNA synthesis in Bacilli

Organism	Antibiotic added*	[^{14}C] uracil incorporated into RNA		[^{3}H] thymidine incorporated into DNA	
		cpm	Relative	cpm	Relative
B. brevis ATCC 8185	None	11,330	(100)	12,760	(100)
B. brevis ATCC 8185	Rifampicin	12	0	3,160	25
B. brevis ATCC 8185	Streptolydigin	4	0	422	3
B. brevis R15	None	1,791	(100)	13,750	(100)
B. brevis R15	Rifampicin	626	35	10,750	78
B. polymyxa	None	922	(100)	21,470	(100)
B. polymyxa	Rifampicin	5	0	15,410	72
B. polymyxa	Streptolydigin	5	0	12,100	56
B. licheniformis	None	381	(100)	3,069	(100)
B. licheniformis	Rifampicin	0	0	2,050	67
B. licheniformis	Streptolydigin	0	0	2,294	75
B. subtilis	None	7,043	(100)	1,741	(100)
B. subtilis	Rifampicin	472	7	511	29

*Antibiotics were added as alcoholic solutions at the following concentrations: Rifampicin, 2 μg/ml; streptolydigin, 20 μg/ml.

RESULTS AND DISCUSSION

As shown in Table I, the incorporation of [^{14}C] uracil into RNA by exponentially growing cultures of several Bacillus species was strongly inhibited by rifampicin and streptolydigin, agents that are known to interact specifically with RNA polymerase (21, 22). At the same time, these antibiotics also inhibited the incorporation of [^{3}H] thymidine into DNA. Such a striking inhibition of DNA replication by inhibitors of RNA polymerase had not before been noted in any other bacterial system, and suggests not only that RNA synthesis plays a role in the replication of DNA in Bacilli, but also that this RNA synthesis must be mediated by the conventional RNA polymerase that is the target of rifampicin and streptolydigin. This was supported by the observation that in B. brevis R15, a strain whose RNA polymerase is partially resistant to rifampicin, the synthesis of both RNA and DNA was less sensitive to inhibition by the antibiotic.

As a preliminary to a more definitive study of this possibility, I

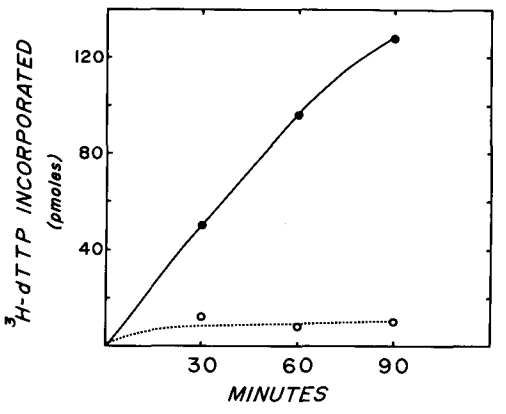

Figure 1. Time course of DNA synthesis in toluene-treated cells of B. brevis. Cells (6 x 10^7) were assayed for the incorporation of [^{3}H] dTTP without (o------o) or with (o———o) 1.5 mM ATP for the times indicated as described under 'Materials and Methods'.

attempted to develop a simpler system in which the dependence of DNA synthesis on RNA polymerase could be studied. Cells made permeable to small molecules by treatment with toluene had already proved very useful in the study of DNA replication in E. coli (20), and since DNA synthesis in B. brevis was especially sensitive to inhibitors of RNA polymerase, I investigated DNA synthesis in toluenized cells from this organism. Figure 1 shows that the incorporation of [^{3}H] dTTP into DNA occurred rapidly and for prolonged times in the presence of ATP, the rate of DNA synthesis per cell being about 80% of that in exponentially growing cultures. In addition to ATP, the incorporation of [^{3}H] dTTP required the presence of all four deoxyribonucleoside triphosphates and Mg^{++} ion (Table II). The requirement for ATP (K_m about 0.1 mM) could be met also by the other ribonucleoside triphosphates. Like DNA replication in E. coli (20), the reaction was inhibited by mercurials; however, it was relatively insensitive to N-ethyl maleimide. DNA synthesis in permeable cells was resistant to rifampicin and streptolydigin at concentrations that were found to completely inhibit RNA polymerase in parallel experiments (not shown). Some inhibition was observed with actinomycin D, but this could have been due to a direct effect on the action of DNA polymerase (23).

Since replication of DNA in growing cells of B. brevis appeared to depend

TABLE II

Requirements for DNA synthesis in toluene-treated cells of B. brevis

Addition or deletion	Relative incorporation of [^{3}H] dTTP
None	(100)*
- dATP	19
- dCTP	17
- dGTP	0.5
- ATP	5
- ATP, + GTP (1 mM)	109
- ATP, + CTP (1 mM)	95
- ATP, + UTP (1 mM)	86
- $MgCl_2$	0.1
+ N-ethylmaleimide (25 mM)	91
+ p-chloromercuribenzoate (0.5 mM)	45
+ p-hydroxymercuriphenylsulfonate (0.5 mM)	40
+ streptolydigin (50 μg/ml)	90
+ rifampicin (50 μg/ml)	115
+ actinomycin D (2 μg/ml)	48

DNA synthesis was assayed in toluene-treated cells (3 x 10^7 cells) as described under 'Materials and Methods', with additions or deletions as indicated.

*29 pmoles [^{3}H] dTTP incorporated.

TABLE III

Inhibition by streptolydigin of DNA synthesis in toluene-treated cells of B. brevis

Treatment	Time of storage	pmoles (^{3}H)TTP incorporated*		% Inhibition
		- Stln	+ Stln	
None	0 weeks	57	55	3
None	2 weeks	71	57	25
None	4 weeks	68	30	55
Preincubation	0 weeks	68	66	3
Preincubation	2 weeks	41	21	49
Preincubation	4 weeks	42	10	76

Toluene-treated cells were kept at -20° for the time indicated, with or without prior incubation under conditions of DNA synthesis as described under 'Materials and Methods', and were then assayed for the ATP-dependent incorporation of (^{3}H)TTP into DNA without and with 50 μg/ml of streptolydigin (Stln).

*Blank values (<3 pmoles) obtained in the absence of ATP have been subtracted.

on the activity of RNA polymerase (Table I), it was puzzling that DNA synthesis in toluenized cells, which resembles in many respects replicative synthesis in toluenized cells of E. coli (20), was completely refractory to inhibitors of RNA polymerase. If we assume that the function of RNA polymerase in replication is to provide primers for the initiation of DNA chains, a possible explanation might be that the amount of RNA primers already present in permeable cells of B. brevis is sufficient to support the limited amount of DNA synthesis seen in this system. In this case, depletion of the cells of unstable RNA should make DNA synthesis sensitive to inhibitors of RNA polymerase. Table III shows that storage of toluene-treated cells at -20° for prolonged periods of time had little effect on the rate of ATP-dependent DNA synthesis but led to a progressive sensitization to inhibition by streptolydigin. This effect could be considerably enhanced if the cells were permitted to engage briefly in DNA synthesis immediately after toluene treatment. Similar experiments with rifampicin showed that sensitivity to this antibiotic developed more slowly than to streptolydigin. After storage at -20° for 4 weeks, rifampicin inhibited ATP-dependent DNA synthesis by 16% or, if the cells were incubated under conditions of DNA synthesis before storage, by 30%. That these effects were not merely a consequence of exposure to low temperatures was shown by the fact that storage at -70° for one year did not lead to increased sensitivity to streptolydigin (not shown). These observations suggest that at -20° a slow degradation of RNA may occur which makes DNA synthesis more dependent on the synthesis of new RNA primers and that this dependence is enhanced by a brief period of prior DNA synthesis. According to this idea, initiation of new RNA chains will be necessary only after the primer fragments have been completely degraded, and sensitivity to rifampicin should therefore develop more slowly than sensitivity to streptolydigin, in agreement with my observations.

In conclusion, the results presented in this paper suggest that DNA replication in Bacilli, unlike in E. coli, depends on the function of the rifampicin- and streptolydigin-sensitive RNA polymerase. DNA synthesis in Bacilli

may thus be considerably less complex than the replication of the E. coli chromosome and rather resembles the more simple system of bacteriophage M13 replication. At the same time, these observations imply that, in Bacilli, RNA polymerase must have a dual function, the transcription of specific genes and the participation in DNA replication. This might explain the puzzling observation that rifampicin, but not actinomycin D, effects a rapid viability loss of dividing cells of B. subtilis, which had originally been interpreted in terms of a second function of RNA polymerase, not involving mRNA synthesis, essential for the survival of dividing cells (24). The system for RNA polymerase-dependent DNA synthesis in toluenized cells of B. brevis, described in this paper, should facilitate the investigation of the role of this enzyme in DNA replication.

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