THE EFFECT OF RIFAMPIN ON THE PRODUCTION OF β22 PHAGE BY BACILLUS SUBTILIS

H. Ernest Hemphill[1] and H. R. Whiteley[2], Department of Microbiology, University of Washington, Seattle, Washington
and
Lyle R. Brown[3] and Roy H. Doi[4], Department of Biochemistry, University of California, Davis, California

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The RNA polymerase which transcribes phage β22 following infection of Bacillus subtilis retains the rifampin sensitivity of the host cell enzyme. Competition-hybridization experiments show that several populations of phage-specific RNA molecules are produced during infection. The ability of rifampin to prevent the maturation of phage β22 is partially lost as soon as the complement of RNA species is qualitatively complete; however, the addition of the antibiotic continues to depress the yield of phage until shortly before lysis, indicating that the quantity of the late RNA species is correlated with the yield of phage.

Investigations of RNA synthesis in mutant bacteria resistant to rifamycin-type antibiotics have led to the conclusion that phage transcription is mediated by an enzyme that retains the antibiotic sensitivity of the host RNA polymerase. Thus, synthesis of T_4-RNA was inhibited by streptovaricin (Mizuno and Nitta, 1969) or rifampicin (Haselkorn, et al., 1969) in sensitive strains of Escherichia coli but not in strains resistant to these antibiotics. In sensitive strains, production of RNA was inhibited throughout the phage cycle (Mizuno and Nitta, 1969); synthesis of specific late proteins (lysozyme and the proteins coded by genes 12 and 34) was inhibited 95% by addition of rifampicin 10 minutes after infection (Haselkorn, et al., 1969). Similarly, synthesis of RNA was inhibited in rifamycin-sensitive strains of Bacillus subtilis but not in resistant strains (Geiduschek and Sklar, 1969). Replication of phage SP01, however, was completely inhibited if rifamycin was added 2 minutes after phage infection but not if added 12 minutes after infection. Geiduschek and Sklar (1969) proposed that insensitivity to the antibiotic

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may indicate that "late" RNA had been synthesized by 12 minutes.

We have been studying the time of synthesis of "early" and "late" phage-specific RNA during the infection of B. subtilis by another virulent phage, β22. In the course of these investigations we have found that the production of phage is sensitive to inhibition by rifampin (also called rifampicin*) until populations of "late" RNA have been produced.

Materials and Methods

Cultures of B. subtilis, strains 168 or SB-11, were grown overnight with shaking to a density of approximately 3 x 10^8 cells/ml in SCM medium containing 0.2% glucose and 0.01% casein hydrolysate (Yehle and Doi, 1967) and infected with β22 phage at a multiplicity of 4. Antiserum to β22 was added 10 min after infection in the experiment shown in Fig. 1. Lysis of both host strains occurred at the same time. Intracellular phage was determined after choloroform treatment; phage assays were performed as described by Yehle and Doi (1967).

The ability of cultures to incorporate H^3-5-uridine was measured by pipetting one aliquot from an infected culture into a tube containing H^3-5-uridine to give a final concentration of 0.4 μC/ml and 10 μg/ml. After 3 min of incubation at 37°, trichloracetic acid (TCA) was added, the precipitated material was collected on a filter and the radioactivity was determined in a scintillation counter using a toluene-based scintillant. Another aliquot of the culture was pipetted into a tube containing 25 μg rifampin/ml (we wish to thank Dr. E. R. Newman of Dow Chemical Co. and Dr. Justus Gelzer of Ciba Pharmaceutical Co. for providing the rifampin) and after 1.5 min, H^3-5-uridine was added to give a concentration of 0.4 μC/ml and 10 μg/ml. The reaction was stopped after 3 min by adding TCA and the TCA-precipitable radioactivity was determined. DNA synthesis was followed by adding H^3-thymidine (0.2 μC/ml and 10 μg/ml) immediately after phage infection and determining the TCA-precipitable radioactivity as a function of time.

*The structure and mode of action of rifampicin and other rifamycin-type antibiotics have been discussed by Wehrli and Staehelin (1969).

Pulse-labeled RNA was prepared in the following manner: cultures were grown to 2.5 x 10^8 cells/ml and infected at a multiplicity of 10. At various times after infection 1 μC/ml (0.01 μg/ml) of H^3-5-uridine was added, the cells were incubated with shaking for 5 min at 37°, the culture was poured over ice, cells were harvested by centrifugation and RNA was extracted as described by Mizuno and Whiteley (1958). β22 DNA was prepared according to Davidson and Freifelder (1962). Competition-hybridization studies were done by the method of Gillespie and Spiegelman (1965). Reaction mixtures contained 4 to 8 μg of the desired pulse-labeled RNA (specific activity 930-2020 cpm), varying quantities of unlabeled 15 to 40 min competitor RNA and 15 μg DNA adsorbed to two 4 mm diameter B-6 membrane filters. These were incubated for 18 hours at 63° in a total volume of 0.4 ml of 2 x SSC (SSC is 0.15 M NaCl-0.015 M sodium citrate).

Results and Discussion

The ability of B. subtilis to incorporate H^3-uridine during infection with phage β22 and the effect of rifampin on this incorporation are illustrated in Fig. 1. In the absence of the antibiotic, incorporation could be divided into 3 phases: an initial decrease immediately after infection, a period of more gradual decrease until 16-20 min after infection, and a period of increase which continued until incorporation reached a value approximatley 2/3 of the level found in uninfected cells. In agreement with studies on other phage infections (Mizuno and Nitta, 1969; Haselkorn, et al., 1969; Geiduschek and Sklar, 1969), rifampin was found to inhibit incorporation at all times during the infection cycle. Incorporation by rifampin-resistant mutants (data not shown) was not afftected by a concentration of 10 μg rifampin/ml in either infected or non-infected cells. These results indicate that the rifampin-sensitive component of the host polymerase functions throughout phage infection.

Although rifampin inhibited RNA synthesis at all times during infection by β22, Table 1 shows that production of infective centers depended on the time of

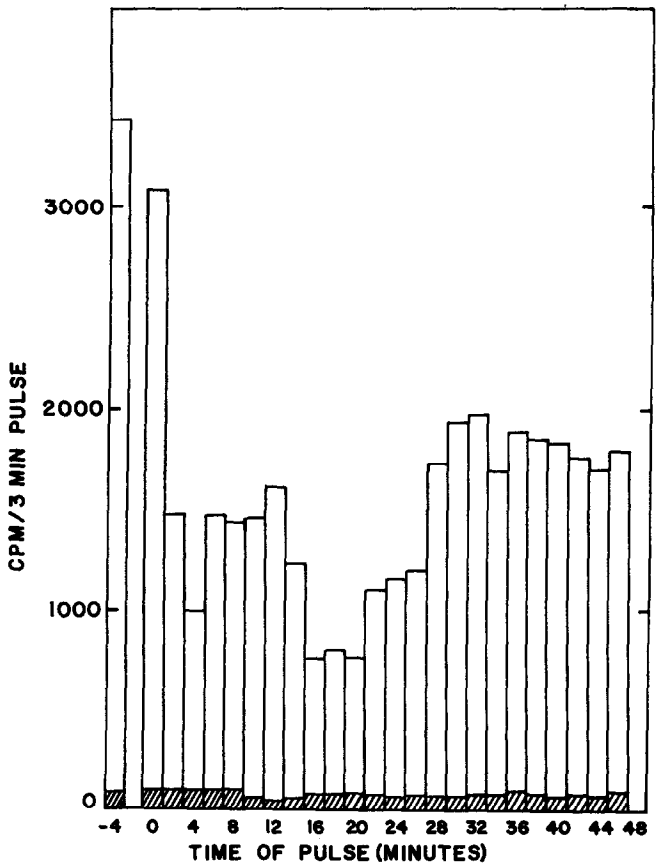

Figure 1: H^3-5-uridine incorporation in the presence and absence of rifampin by B. subtilis infected with phage β22.
Open and closed bars show incorporation during a 3 min pulse in the absence and presence, respectively, of rifampin. Phage was added to both cultures at zero time. Cells were pulsed at 2 min intervals as described in Materials and Methods.

addition of the antibiotic. Up to 30 min after infection, inhibition of phage production was >99%; thereafter, progressively more phage was produced in the presence of the drug although some inhibition was noted even when rifampin was added a very short time prior to lysis. Since antibiotics of this type are thought to function as inhibitors of initiation of RNA synthesis (Wehrli, et al., 1968; di Mauro, et al., 1969), it appears that maximal phage production may require continued initiation of new molecules of RNA until late in the infection and well after the appearance of intracellular phage.

Table 1

Formation of phage β22 infective centers when rifampin was added at different times after infection.

Time of RFP Addition	PFU 80 min after infection	percent yield
--	9.2×10^6	100.0
15	4.0×10^4	0.4
20	2.3×10^4	0.3
25	4.5×10^4	0.4
30	3.4×10^4	0.3
35	3.0×10^5	3.3
40	1.3×10^6	14.1
45	2.3×10^6	25.0
50	6.1×10^6	66.0
55	6.8×10^6	73.9
60	7.1×10^6	77.2

At the times indicated, aliquots of an infected culture were removed, 10 μg rifampin (RFP)/ml were added, the culture was incubated for 80 min and the plaque-forming units (PFU) were determined.

An increase in intracellular phage (Fig. 2) could be detected at approximately the same time as the onset of rifampin-insensitivity. This was preceded by an increase in the incorporation of H^3-thymidine, presumably reflecting synthesis of phage DNA. DNA/DNA hybridization studies (data not shown) demonstrated that phage DNA synthesis begins prior to the 20th min of the infection cycle, and that it is the only DNA replicated after this time. This increase in phage DNA (either in amount of DNA or the availability of a different template for RNA polymerase) may account for the increase in incorporation of H^3-uridine into RNA noted during the second half of the infection cycle (Fig. 1), although other explanations are also possible.

Lastly, the onset of insensitivity of phage production of rifampin was correlated in time with the synthesis of late phage-specific RNA. Figs. 3A and 3B show: 1) that different kinds of RNA molecules were produced throughout infection (a detailed analysis of these curves will be presented elsewhere), 2) that these could not be separated into two simple classes corresponding to "early"

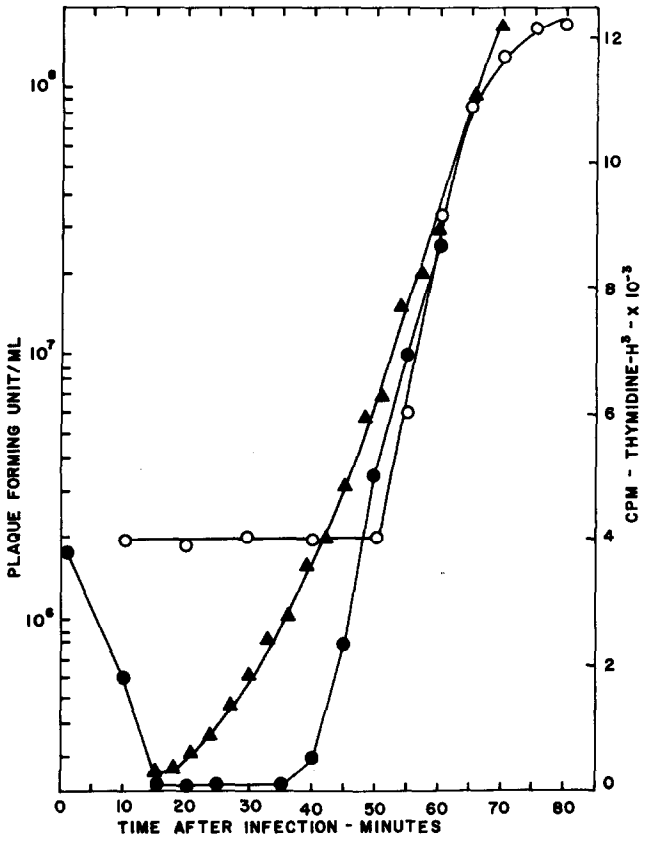

Figure 2: Incorporation of H^3-thymidine, production of intracellular phage and formation of infective centers following infection of B. subtilis with phage β22.
Procedures as described in Materials and Methods.

O——O = infective centers; ●——● = intracellular phage; ▲——▲ = cpm H^3-thymidine incorporated.

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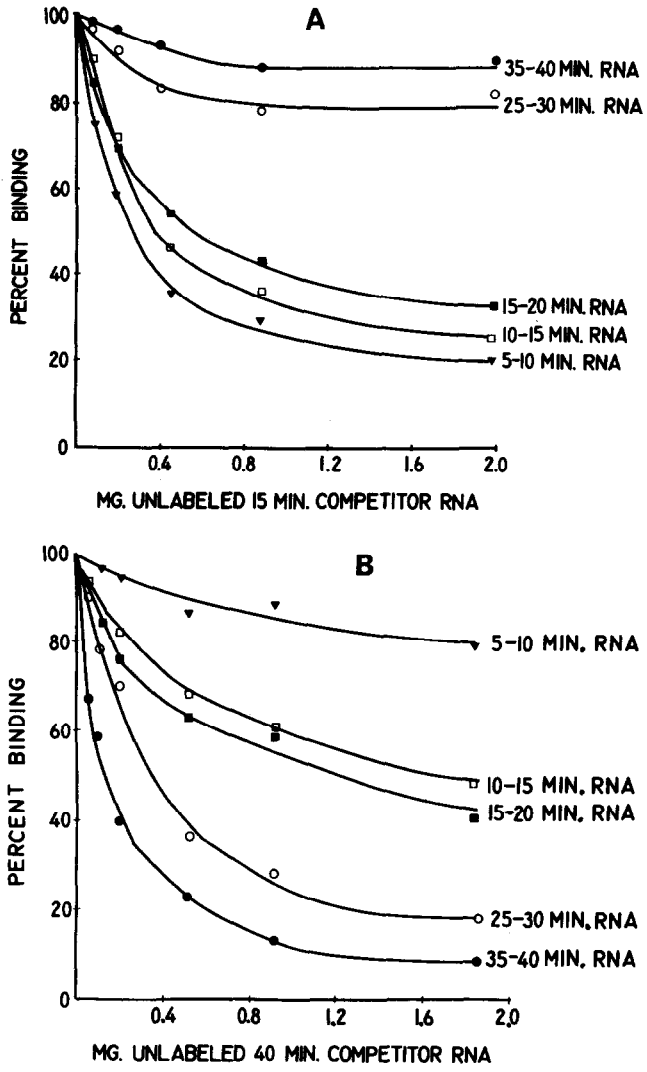
and "late" RNA and, 3) that RNA produced during the second half of the infection cycle (after 25 min) was different from that produced early. Much of the RNA characteristic of the later stages of infection (Fig. 3A) was synthesized subsequent to the 15-20 min interval although some early species continue to be produced 40 min after infection (Fig. 3B). It is not known whether phage DNA synthesis, which begins in the first third of the infection cycle is directly correlated with the transcription of late RNA populations. However, synthesis of some of these late species was not initiated until after the



Figures 3A and 3B: Binding of H^3-phage RNA to β22 DNA in the presence of increasing amounts of unlabeled competitor RNA.

Competitor RNA was extraced from phage-infected cells 15 min after infection (Fig. 3A) and 40 min after infection (Fig. 3B). H^3-RNA preparations were labeled as described in Materials and Methods.

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beginning of phage DNA replication. Once RNA characteristic of late stages (35-40) was transcribed, production of mature phages could be expected even in the presence of rifampin. The period of rifampin sensitivity, therefore, defines the time required for qualitative transcription of all species of RNA needed for phage production.

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