Effect of Nalidixic Acid on PBS2 Bacteriophage Infection of *Bacillus subtilis*

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Infection of *Bacillus subtilis* by PBS2 phage, whose DNA contains uracil instead of thymine, is relatively unaffected by low concentrations of nalidixic acid which severely inhibit *B. subtilis* DNA synthesis. High concentrations of nalidixic acid do inhibit PBS2 DNA synthesis, but more severely reduce the burst size of PBS2 infections. Hydroxyurea blocks PBS2 DNA synthesis, preventing progeny phage production.

The antibiotic, nalidixic acid (NAL), is a relatively specific inhibitor of DNA synthesis in *Bacillus subtilis*, leading to "unbalanced growth" in which cells continue to elongate and to synthesize RNA and protein (3). NAL does not inhibit DNA polymerase activity in crude extracts or membrane fractions of wild-type *B. subtilis* (7). NAL does not appear to block the biosynthesis of deoxyribonucleoside triphosphates (2, 5), and the molecular mechanism of action of NAL remains unknown (7). However, it has been observed by others that DNA synthesis by certain bacteriophages (*Escherichia coli* phages T5, T7, λ, and φR) is strongly inhibited by NAL (1), whereas other bacteriophages (*E. coli* phages T2 and T4) are only partially inhibited (1), and the *B. subtilis* phages SP01 (4), SP82, SP50, and φ29 (1), and SPP1 (6) are relatively resistant to this antibiotic. It was therefore of interest to determine whether NAL would affect the infection of *B. subtilis* by the phage PBS2, unique because its DNA contains uracil instead of thymine.

Methods for the growth and titering of *B. subtilis* SB19 cells and PBS2 phage, colorimetric DNA determination, premature lysis of infected cells by lysozyme in the presence of chloramphenicol, and assay for PBS2 deoxythymidylate phosphohydrolase (dTMPase) activity have been described (8–11). NAL was obtained from Calbiochem, and hydroxyurea was from Sigma Chemical Co. Fresh solutions of NAL or hydroxyurea were prepared daily using dimethylsulfoxide as the solvent. In all experiments, dimethylsulfoxide was added as 1% of the culture volume for control and drug-treated cells.

The results (Fig. 1) confirm the findings of Cook et al. (3) that NAL prevents DNA synthesis without affecting the increase in optical density of *B. subtilis* culture. Under our conditions, NAL at 50 µg/ml prevented net DNA accumulation for 3 h in uninfected cells. Lower concentrations of NAL stopped DNA synthesis only temporarily, with accumulation resuming at a slower rate after 40 min in 10 µg/ml and after 90 min in 20 µg/ml. It is not known if NAL must be converted to some active metabolite to prevent DNA synthesis (7), but it is possible that NAL or its active metabolite may be slowly inactivated under our conditions of high cell density in rich medium. Restoration of DNA synthesis at these levels of NAL was not apparently observed by Cook et al. (3) and Pedrini et al. (6). Their experiments were done at 5- to 10-fold lower *B. subtilis* cell density than those used here. However, the experiments of Gage and Fujita (4), performed at cell titers of 1.8 × 10⁸/ml, show an incomplete shut-off of DNA synthesis by NAL at 10 to 20 µg/ml. Nonetheless, our experiments indicate that NAL is an effective inhibitor of cellular DNA synthesis.

In comparison to host DNA synthesis, the results shown in Fig. 2 indicate that PBS2 phage DNA synthesis was relatively resistant to low concentrations of NAL. During normal infections the synthesis of host DNA stops after PBS2 infection, and phage DNA synthesis begins after 20 min (see reference 9). The addition of NAL at 10 µg/ml or 20 µg/ml at the time of infection did not appreciably affect the rate of phage DNA synthesis. It caused only a slight delay in the onset and a small reduction (10 and 20%, respectively) in the extent of phage DNA synthesis. Induction of the viral dTMPase occurred at the normal time (11) and at almost
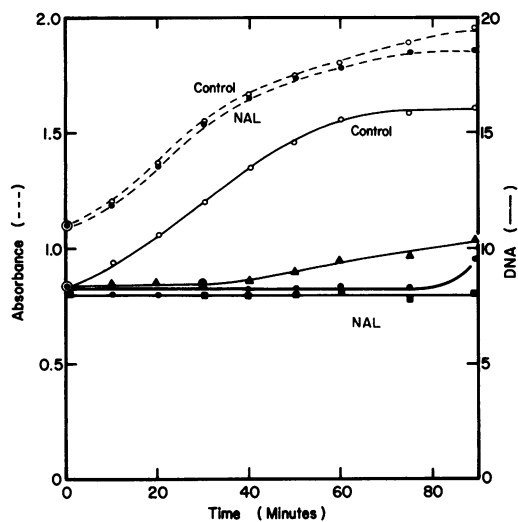
FIG. 1. *Effect of nalidixic acid on cell growth and DNA synthesis in B. subtilis. Cells were grown at 37 C with vigorous shaking in Penassay broth (Difco) to an absorbance of 1.1 at 660 nm in a 1-cm cuvette. Nalidixic acid (NAL) was added to give a final concentration of 0 (○), 10 (▲), 20 (●), or 50 (■) µg/ml. The culture absorbance (dashed lines) and DNA concentration in micrograms per milliliter (solid lines) were determined at the indicated times after addition of nalidixic acid.*
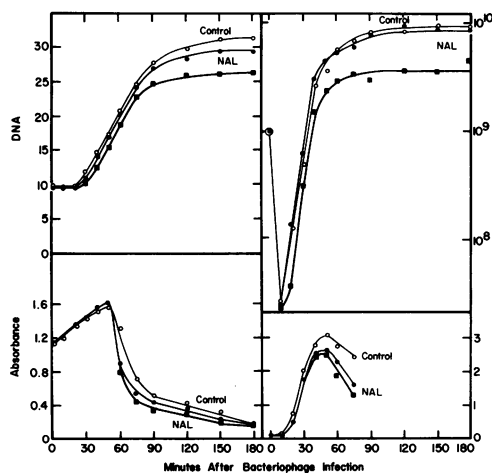
TABLE 1. *Effect of nalidixic acid on the burst size of PBS2 bacteriophage infections of B. subtilis*

Time after infection of nalidixic acid addition (min)	Burst size[a]			
	0 µg/ml[b]	10 µg/ml	20 µg/ml	50 µg/ml
−15		26	15	1
−5		39	16	3
0	50	39	27	4
5		48	26	5
15		55	35	8
30		46	34	33
45			46	35
60		43	43	39

[a] Cells were grown as in Fig. 1 to an absorbance at 660 nm of 1.0 (2×10^8 cells per ml). They were treated at the indicated time before or after infection with the indicated concentration of nalidixic acid. Infections were performed at a multiplicity of 5 phage per cell. At 2 h after infection (when lysis was complete), cultures were titered for phage to determine the burst size (phage produced per original viable cell). The burst sizes shown represent actual phage production, since premature lysis of infected cells at 15 min after infection showed that a true eclipse had occurred, with less than 0.2 free phage remaining per original cell.

[b] Nalidixic acid concentrations.

FIG. 2. *Effect of low levels of nalidixic acid on PBS2 infection of B. subtilis. Cells grown as in Fig. 1 were infected at a multiplicity of 5 phage per cell. At the same time, nalidixic acid (NAL) was added at a final concentration of 0 (○), 10 (●), or 20 (■) µg/ml. Aliquots were removed and processed as described previously (10) to determine the DNA concentration (micrograms per milliliter), absorbance at 660 nm, total phage (plaque-forming units per milliliter), and deoxythymidylate phosphohydrolase (dTMPase) activity (micromoles of phosphate released in 15 min at 37 C per milligram of protein).*

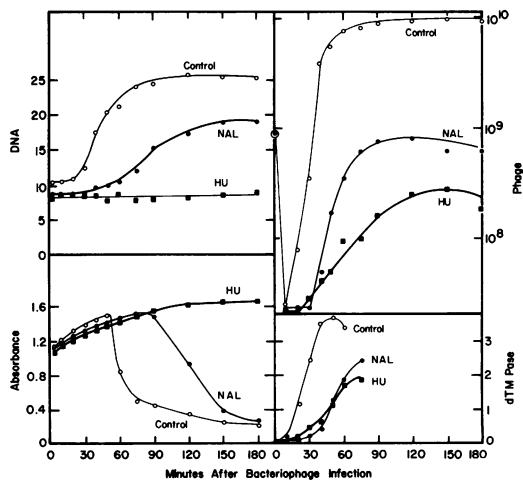
FIG. 3. *Effect of a high concentration of nalidixic acid or hydroxyurea on PBS2 infection of B. subtilis. Cells grown as in Fig. 1 were treated with dimethyl-sulfoxide alone (○), nalidixic acid (NAL) at 50 µg/ml (●), or hydroxyurea (HU) at 0.16 M (■). After 5 min PBS2 phage were added at a multiplicity of 5 per cell, and samples were removed for determinations as in Fig. 2.*

normal levels in the presence of NAL. Lysis of infected cells began after 50 min and proceeded somewhat more rapidly in the presence of NAL.

Figure 2 shows that the production of viable progeny phage was delayed only slightly by NAL, and the final phage yields were 80 and 50% of the control values when NAL was added at 10 μg/ml and 20 μg/ml, respectively. Thus, PBS2 infection of *B. subtilis* is relatively unaffected by concentrations of NAL which drastically inhibit DNA synthesis in uninfected cells.

The effect of the time of addition of NAL relative to PBS2 infection was determined. Table 1 indicates that 10 μg of NAL per ml, added 15 min before phage, resulted in a 50% reduction in the burst size. Later addition of this concentration of NAL allowed 80 to 100% of the normal phage production (50 phage per cell). The presence of 20 μg of NAL per ml was more inhibitory, causing a 70% reduction in burst size when added 15 min before phage. Progressively later additions of NAL at 20 μg/ml allowed increasingly higher phage yields. At 50 μg/ml, NAL added 15 min or 5 min before infection inhibited phage production by 98 or 94%, respectively. The addition of NAL at 50 μg/ml at 30 to 60 min after phage infection (when phage DNA synthesis and virus assembly had begun) had much less effect on the final burst size. Similar results have been obtained by one-step growth experiments performed as described by Baird et al. (1), showing that NAL neither affects phage adsorption to cells nor does it kill free phage.

To examine further the inhibition of phage production caused by a high concentration (50 μg/ml) of NAL added before infection, the experiment described in Fig. 3 was performed. NAL at 50 μg/ml delayed the onset of phage assembly and reduced the final burst size by 92%. NAL slowed the rate of phage DNA synthesis and reduced its final level to two-thirds of the control value. Similar delays and partial inhibition of phage DNA and virus production by high concentrations of NAL have also been observed by others using different *B. subtilis* phages (1, 4, 6). NAL at 50 μg/ml inhibits bacterial RNA and protein synthesis (3, 13). This effect can explain the delay and the decrease in the rate of viral enzyme induction and cell lysis seen in Fig. 3. Unlike 6-(*p*-hydroxyphenylazo)-uracil (10), high concentrations of NAL do not give specific inhibition of bacterial DNA synthesis, but appear to have other unrelated and perhaps nonspecific effects. It is apparent that PBS2 phage DNA synthesis is much more resistant to NAL than *B. subtilis* DNA synthesis.

The effects of adding 0.16 M hydroxyurea before PBS2 infection are shown in Fig. 3.

Hydroxyurea, another inhibitor of bacterial DNA synthesis, acts primarily by blocking ribonucleotide reduction to deoxyribonucleotides (2, 5, 12). In PBS2 phage-infected *B. subtilis*, hydroxyurea prevented the net accumulation of DNA and prevented lysis. In addition, hydroxyurea reduced by twofold the rate and level of PBS2 dTMPase induction, and phage production was reduced to two phage per cell. We suggest that if PBS2 phage induces a new ribonucleotide reductase, as is known for *E. coli* phage T4 (14), then the PBS2-induced reductase is also sensitive to hydroxyurea, which prevents PBS2 DNA replication.

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