Effect of Nalidixic Acid on Deoxyribonucleic Acid Synthesis in Bacteriophage SPO1-infected Bacillus subtilis

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The effect of nalidixic acid on deoxyribonucleic acid (DNA) synthesis in Bacillus subtilis cells infected with bacteriophage SPO1 was studied. Nalidixic acid had little inhibitory effect on SPO1 DNA synthesis at concentrations that drastically inhibited B. subtilis DNA synthesis. Inhibition of DNA synthesis, appropriate to the concentration used, was imposed within 1 min after addition of nalidixic acid, suggesting that it acts directly on DNA synthesis in both infected and uninfected cells. The SPO1 DNA synthesized in the presence of high concentrations of nalidixic acid had a density characteristic of normal SPO1 DNA and was packaged into viable progeny phage particles, but its rate of synthesis was reduced and bacterial lysis was delayed.

Nalidixic acid (1,4-dihydro-1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) is a selective inhibitor of deoxyribonucleic acid (DNA) synthesis in growing Escherichia coli (4, 5) and Bacillus subtilis (2) cells. It does not appreciably affect bacterial ribonucleic acid (RNA) or protein synthesis at concentrations that strongly inhibit bacterial DNA synthesis, although the basis for this selectivity and the mechanism of its action are unknown.

In this paper, we demonstrate that nalidixic acid has a weak inhibitory effect upon phage SPO1 DNA synthesis, at concentrations that drastically inhibit uninfected B. subtilis DNA synthesis.

MATERIALS AND METHODS

Bacteria. All experiments were performed with B. subtilis HA101, a multiple auxotroph requiring histidine, methionine, and leucine, and nonpermissive for a class of phage SPO1 mutants (7). HA101 was generously provided by S. Okubo.

Phage. SPO1 wild type (6) and SPO1 Sus F30 were used. Sus F30, a suppressor mutant of SPO1 isolated by one of us (D.J.F.), grows on the permissive host HA101-B, an su+ derivative of HA101 (7). Sus F30 does not synthesize phage DNA (DO) in nonpermissive hosts (HA101 or 168 M).

Media. HML medium contained (per liter): glucose, 5 g; L-histidine, 50 mg; L-methionine, 50 mg; L-leucine, 50 mg; NBS, 5 ml; and minimal salts medium (1), 995 ml. L medium is HML medium without histidine and methionine. NBS is NY medium without yeast extract (6).

Phage preparation. Wild-type phage were prepared in CHT-50 medium (1), supplemented with 5 × 10−4 m CaCl2 and 0.1% casein hydrolysate, essentially according to standard procedures (6). Sus F30 stocks were prepared on the HA101-B host in L medium.

Nalidixic acid. Nalidixic acid was a generous gift of S. Archer, Sterling-Winthrop Research Institute. Stock solutions were made by dissolving the agent in dimethyl sulfoxide (DMSO) at a concentration of 6.0 mg/ml, and were stored frozen. Prior to use, further dilutions were made into growth medium at 37°C. Concentrations were standardized spectrophotometrically (Ext 260 = 1.1 × 104 cm−1/g). All cultures of any single experiment contained uniform amounts of DMSO (<0.8%) during the period of exposure to nalidixic acid. There was no observable effect of DMSO on infected or infected cells at the concentration used.

Phage infection. All experiments were carried out at 37°C. Bacteria were grown to 108 to 1.8 × 109 cells/ml and were infected at multiplicities of 6 to 10 with SPO1. At 37°C, in HML medium, the bacterial doubling time was 40 min, the SPO1 eclipse period was 28 min, cell lysis began at 35 min, and the average burst was about 80 phage per infectious center.

Incorporation of labeled adenine into DNA. Incorporation of adenine-8-14C into alkali-stable, trichloroacetic acid-precipitable material was used as a measure of DNA synthesis. For most experiments, 1-ml samples were added directly to 1 ml of 1 m KOH and incubated at 37°C for at least 12 hr. Each sample was
then precipitated with 0.5 ml of 50% trichloroacetic acid containing 1 mg of unlabeled adenine per ml, chilled, and diluted further with 2 ml of 5% trichloroacetic acid. Fractions from CsCl gradients were added to 1 ml of 0.5 M KOH, with similar subsequent treatment. Precipitates were collected on nitrocellulose membrane filters, washed with 5% trichloroacetic acid containing 100 µg of unlabeled adenine per ml, dried, and counted in a scintillation spectrometer in Liquifluor-toluene.

CsCl density gradient centrifugation. At appropriate times, 5 ml of labeled culture was poured onto 5 ml of frozen minimal salts medium (1). Sodium azide was added to 0.01 M, and the cells were separated by low-speed centrifugation. The cells were then suspended in 2 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate) supplemented with 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5) and 0.01 M KCl. Lysozyme was added to 300 µg/ml, and the preparations were quick frozen, thawed, and incubated at 37 C; sarkosyl was added to 1.5%, which caused clearing of the cell suspension. Appropriate quantities of saturated CsCl solution were added, and tubes were centrifuged in an SW39 rotor for 72 hr at 61,000 X g and 22 C. Refractive indices were determined on a representative number of fractions, and then all samples were treated with KOH and trichloroacetic acid as previously described.

One-step growth experiment. HA101 cells (1.8 X 10^9/ml) were infected with wild-type SPO1 at a multiplicity of 6 and aerated at 37 C, at appropriate dilutions. Parallel experiments were carried out in the presence and absence of nalidixic acid (50 µg/ml). Intracellular phage were assayed by the method of Geiduschek and Sklar (Nature, in press). Extracellular phage and infectious centers were sampled directly at various times from the appropriately diluted growth tubes, and were plated with indicator bacteria.

RESULTS

The effect of nalidixic acid on DNA synthesis in uninfected and infected cells was measured by incorporation of adenine-8-14C into alkali-stable, trichloroacetic acid-precipitable material. Alterations in rate or extent of incorporation were assumed to reflect similar alterations in DNA synthesis.

In the first set of experiments, cells were preincubated in nalidixic acid for 5 min before the addition of label, or label and phage. In uninfected cells, bacterial DNA synthesis was strongly inhibited by nalidixic acid (Fig. 1a), as has been shown previously (2), but DNA synthesis in SPO1-infected cells was inhibited to a lesser extent (Fig. 1b). For example, 5 µg of nalidixic acid per ml inhibited the total incorporation of label into DNA of uninfected cells at 60 min by 75%, but had no appreciable effect on incorporation into infected cells. A concentration of 25 µg/ml inhibited total DNA synthesis in uninfected cells by about 95% at 60 min, but inhibited total DNA synthesis in infected cells only by about 30%.

Inhibition of DNA synthesis in infected cells, when measured at 60 min, was due not only to a depression of the rate of synthesis but also to a delay in the onset of phage DNA synthesis. This delay is more clearly demonstrated in experiments presented below.

The DNA synthesized after phage infection in the presence of nalidixic acid has been characterized in the following ways. (i) CsCl density gradient analysis of pulse-labeled DNA synthesized early in SPO1 infection of B. subtilis 168 M, in the absence of nalidixic acid, showed that host DNA synthesis continues for several minutes after infection but is shut off before the onset of phage DNA synthesis (D. L. Wilson, personal communication). (ii) An SPO1 mutant, Sus F30, deficient in DNA synthesis (DO), shuts off host DNA synthesis but synthesizes no viral DNA in nonpermissive hosts (Fujita and Gage, unpublished data). The patterns of DNA synthesis after infection of the nonpermissive host HA101 by Sus F30 and by wild-type SPO1 are compared in Fig. 2. Incorporation was virtually identical in
FIG. 2. Incorporation of label into the DNA of B. subtilis HA101 cells infected with SPO1 Sus F30 and wild-type SPO1. Adenine-8-14C (15 μg/ml, 0.005 μc/μg) and phage (multiplicity of infection = 10) were added after a 5-min preincubation of cells in 50 μg of nalidixic acid per ml, or in its absence. No nalidixic acid: curve A (△), Sus F30-infected; curve B (□), wild-type SPO1-infected; curve C (▲), Sus F30-infected; curve D (●), wild-type SPO1-infected.

The effect of preincubation in nalidixic acid on subsequent phage DNA synthesis was examined by the addition of phage and labeled adenine to cells after various periods of exposure to the

curve, thus eliminating consideration of the delay in the onset of synthesis.

In addition to showing the considerable difference in inhibitory effect of nalidixic acid on B. subtilis and SPO1 DNA synthesis, these results lead to two other important conclusions: (i) the inhibitory effect of nalidixic acid upon active DNA synthesis is expressed fully within 1 min after addition of the drug (Fig. 3), and (ii) the extent of inhibition of the ultimate rate of adenine incorporation into phage DNA is the same regardless of whether the inhibitor is added before infection or after phage DNA synthesis has begun (Fig. 4, curve B).

To determine the rapidity with which inhibition by nalidixic acid is established, the agent was added to infected and uninfected cells actively incorporating label into DNA. In both cases, the inhibitory effect was expressed within 1 min after addition of the drug (Fig. 3), and the inhibition of bacterial DNA synthesis was again much greater than the inhibition of phage DNA synthesis. Significant inhibition in uninfected cells was evident even with concentrations of the inhibitor as low as 1 μg/ml.

Relative incorporation rates, measured at various inhibitor concentrations and plotted as a function of concentration, serve to illustrate the differing extent of inhibition of phage and bacterial DNA synthesis by nalidixic acid (Fig. 4). Incorporation rates in uninfected cells were determined soon after addition of nalidixic acid to minimize effects of continued cell division. Inhibition in infected cells was determined by comparison of the linear portions of each incorporation

FIG. 3. Effect of addition of nalidixic acid to uninfected and SPO1-infected B. subtilis HA101 cells actively incorporating labeled adenine into DNA. (a) Uninfected cells: adenine-8-14C (15 μg/ml, 0.005 μc/μg) was added at t = 0, and nalidixic acid at t = 16.33 min. (b) Infected cells: adenine-8-14C (15 μg/ml, 0.005 μc/μg) was added 2 min before infection with wild-type SPO1 (multiplicity of infection = 10) with nalidixic acid added at 24 min after infection. Visible clearing of all cultures began at 40 min after infection. Nalidixic acid concentrations: □, none (control); △, 1 μg/ml; ●, 3 μg/ml; ○, 5 μg/ml; ▲, 10 μg/ml; □, 20 μg/ml; X, 50 μg/ml.
inhibitor (Fig. 5). Preincubation for 5 min resulted in a delay of about 9 min in the onset of phage DNA synthesis as compared with a control infection in the absence of nalidixic acid. Longer exposure to nalidixic acid before infection caused no further effect on the time of onset or on the subsequent rate of phage DNA synthesis, except for a small additional delay of about 5 min in the onset of synthesis in cells exposed for more than 60 min (Fig. 5b).

Implicit in these results is evidence for two effects of nalidixic acid on DNA synthesis in infected cells. Most obvious is the direct decrease in rate of phage DNA synthesis, which was independent of time of addition of the inhibitor (Fig. 4, curve B). The other effect, which occurred only if nalidixic acid was present for a period before active phage DNA synthesis, resulted in a delay in the onset of that synthesis (Fig. 5b).

Similar experiments performed at a low multiplicity of infection (0.7) showed that the differential inhibition of rate of DNA synthesis was independent of the input ratio of phage to bacteria (compare Fig. 6, curves C and D, with Fig. 3b). However, the delay in onset of phage DNA synthesis resulting from nalidixic acid addition before infection was longer than at higher multiplicities (compare Fig. 6, curve E, with Fig. 1b).

In other experiments (data not shown), nalidixic acid added at the time of normal onset of phage DNA synthesis caused a depression in the rate of DNA synthesis, but did not result in a delay in its onset. In addition, visible lysis was delayed to an extent dependent upon inhibitor concentration in infections made in the presence of nalidixic acid, but no such delays were observed when inhibitor additions were made after phage DNA synthesis had begun. Indications of this are evident in Fig. 3b and Fig. 5. Addition of nalidixic acid to cells that had already started to replicate phage DNA slowed the rate of that

![Graph](http://jb.asm.org/)
cells infected with nalidixic acid at the infection = 20 (15-8-14C in infection. Lowered incorporation plateaus were reached, but did not delay lysis, resulting in a plateau (Fig. 3b). Nevertheless, preincubation of cells in 43 μg of nalidixic acid per ml delayed lysis so that higher incorporation plateaus were eventually reached in spite of the delay in onset of replication and the depressed rate of DNA synthesis (Fig. 5). Although the depression in rate of SPO1 DNA synthesis was dependent only upon the concentration of nalidixic acid, both the delay in onset of DNA synthesis and the delay in lysis were dependent also on the time of addition of the inhibitor.

The effect of nalidixic acid on development of viable SPO1 was examined in a “one-step growth” experiment (3). Infection in the presence of 50 μg of nalidixic acid per ml caused a delay of 30 min in intracellular phage appearance, and a delay of 50 min in lysis (Fig. 7). These delays appear to be too long to be ascribed only to the inhibitor's effects on DNA synthesis. The production of viable phage progeny, as illustrated in this experiment, is, needless to say, a most critical test of the biological integrity of the DNA synthesized in the presence of the inhibitor.

SPO1 DNA contains hydroxymethyluracil in place of thymine. Consequently, the DNA of B. subtilis and that of SPO1, although similar in guanine plus cytosine content, have very different densities in CsCl, which allows simple identification of newly synthesized DNA (6). Uninfected cells were labeled, either in the presence or absence of 50 μg of nalidixic acid per ml, for 40 min before addition of phage, and for an additional period after infection. The DNA was then prepared, and was centrifuged to equilibrium in CsCl (Fig. 8a and b). Because of the delay in onset and the depressed rate of SPO1 DNA synthesis in the presence of nalidixic acid, the DNA labeling period following infection was adjusted so that the extent of labeled phage DNA synthesis was

![Graph of labeled DNA synthesis](https://example.com/graph.png)

**Fig. 6.** Effect of nalidixic acid on B. subtilis HA101 cells infected at low SPO1 multiplicity (multiplicity of infection = 0.7). Nalidixic acid, phage, and adenine-8-14C (15 μg/ml, 0.005 μc/μg) additions are indicated on the figure. Curve A (○), uninfected cells; curve B (●), portion of A added to nalidixic acid (50 μg/ml) at 20 min; curve C (△), SPO1-infected cells; curve D (▲), portion of C added to nalidixic acid (50 μg/ml) at 20 min, 20 sec; curve E (■), SPO1-infected cells, nalidixic acid (50 μg/ml) added at 8 min before infection.

**Fig. 7.** One-step growth experiment, showing effect of nalidixic acid on SPO1 development. A culture of HA101 cells was divided, with one part receiving 50 μg of nalidixic acid per ml and one part receiving no nalidixic acid. Each was infected with wild-type SPO1 (multiplicity of infection = 6). Dilutions of 10⁻⁵, 10⁻⁴, 10⁻³, and 10⁻² were made into bubbler tubes and aerated with 50 μg of nalidixic acid per ml where appropriate. Intracellular phage development was assayed at times shown, from the 10⁻⁴ dilutions: ○, none (control); ▲, 50 μg/ml. Extracellular phage and infectious centers were plated directly from the proper dilutions: ○, none (control); ●, 50 μg/ml.
Density gradient analysis of DNA prepared after pulse labeling late in infection in the presence of 50 μg of nalidixic acid per ml is shown in Fig. 8c. There was no label corresponding to the density of bacterial DNA, and all DNA synthesized during this pulse was found at the characteristic SPO1 density.

**DISCUSSION**

These results demonstrate that nalidixic acid has little effect on phage SPO1 DNA synthesis, at concentrations that greatly inhibit *B. subtilis* DNA synthesis. Although nalidixic acid acted only weakly on SPO1 DNA synthesis, two different effects were distinguishable. The first, a slight reduction in the rate of SPO1 DNA synthesis, was observed regardless of whether the drug was added to cells before phage infection, or to infected cells which were actively synthesizing phage DNA. At effective concentrations, it resulted in a depression in the rate of SPO1 DNA synthesis, which was fully expressed within 1 min after drug addition. The rapidity (but not the extent) of this effect upon active phage DNA synthesis was comparable to that upon bacterial DNA synthesis in uninfected cells and suggested that it results from a direct action upon DNA synthesis. The second effect, a concentration-dependent lag in the onset of phage DNA synthesis, was observed when nalidixic acid was added to cells prior to phage infection. However, the ultimate rates of DNA synthesis after the lag period, at various drug concentrations, were comparable to those obtained when the drug was added to infected cells actively synthesizing SPO1 DNA. A delay in the appearance of intracellular phage and a delay in lysis were also observed under conditions which delayed SPO1 DNA synthesis.

The basis of the differential inhibition of *B. subtilis* and SPO1 DNA synthesis is unknown, as is the mechanism of nalidixic acid action upon bacterial DNA synthesis. However, the following results suggest that a difference in permeability to nalidixic acid between infected and uninfected cells is probably not involved: (i) the inhibitory effect was fully imposed with equal rapidity in infected and uninfected cells; (ii) the same reduction in the rate of SPO1 DNA synthesis was observed whether the drug was added to cells before infection, or to infected cells after the onset of phage DNA synthesis; and (iii) bacterial DNA synthesis was inhibited to the same extent after phage infection (before phage-induced host DNA shut off) as in uninfected cells. Since nalidixic acid appears to enter infected cells as rapidly as uninfected cells, and has the same effect on DNA synthesis whether it is added before or after infection, a mechanism attributing the differential effect of

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**Fig. 8.** Density gradient centrifugation of DNA synthesized in the presence of nalidixic acid. A culture of *B. subtilis* HA101 cells was divided into three parts and two (b, c) received 50 μg of nalidixic acid per ml. After 5 min, adenine-8-14C (15 μg/ml, 0.133 μCi/μg) was added to cultures a and b. After an additional 40 min, SPO1 (multiplicity of infection = 10) was added to all three. (a) Control, no nalidixic acid, cells collected 25 min after infection; (b) 50 μg of nalidixic acid per ml, cells collected 60 min after infection; (c) 50 μg of nalidixic acid per ml, adenine-8-14C (6 μg/ml, 0.33 μCi/μg) was added at 50 min after infection, and the cells were collected at 60 min after infection. Refractometrically determined densities in CsCl are shown over arrows.

Similar to the control. Because of these considerations, the relative amounts of label accumulated into the SPO1 DNA density band are not quantitative measures of phage DNA synthesis in the presence and absence of the inhibitor. However, such a quantitative comparison of bacterial DNA synthesis is possible, since the period of synthesis of labeled bacterial DNA is the same in each case, extending from label addition to phage-induced host shut off. Accumulation of labeled bacterial DNA was about 20 times as great in the absence of nalidixic acid as in its presence, which is entirely consistent with the inhibition of bacterial DNA synthesis by 50 μg of nalidixic acid per ml, illustrated in Fig. 1a and 3a.
the drug to a postinfection change in permeability, or to a difference in intracellular drug concentration, seems unlikely. Active catabolism of the drug during infection also seems unlikely, because the reduced rates of DNA synthesis, once established, were constant until cell lysis occurred.

We have no other evidence concerning the basis of the observed differential inhibition, but it should be noted that there are certain differences between normal SPO1 and *B. subtilis* DNA synthesis which may be relevant to this phenomenon. SPO1 DNA contains hydroxymethyluracil instead of thymine. Therefore, in the course of normal infection, alterations are made in *B. subtilis* DNA precursor pathways in order to allow SPO1 DNA synthesis to occur. The enzymes of the phage-specific pathways may be more resistant to the drug than are the host enzymes. It is also possible that SPO1 DNA, like T4 DNA, may have many more replication points than host DNA during active synthesis. Finally, numerous recombination events between members of the SPO1 DNA pool in infected cells might somehow provide a release from inhibition.

The cause of the concentration-dependent delay in the onset of phage DNA synthesis, the other inhibitory effect, is also not fully understood. One possible explanation is that this delay arises from a secondary effect of the drug, such as a partial inhibition of protein synthesis, which has been observed in uninfected *B. subtilis* cells after long exposures to high nalidixic acid concentrations (2). Another possibility is that a partial inhibition of functional SPO1 messenger RNA transcription might occur if the drug interacts with phage DNA molecules. Such an effect occurring early in infection might delay the accumulation of SPO1 gene products necessary for DNA synthesis, resulting in a delay in the onset of phage DNA synthesis. It is of interest that the lag appears to be multiplicity-dependent and is not observed if the drug is added to infected cells after SPO1 DNA synthesis has begun.

Nalidixic acid also caused a delay in the production of intracellular phage, and in cell lysis. The lengths of these delays, at various drug concentrations, were not equivalent to the corresponding delay in the onset of phage DNA synthesis, and were probably influenced by other factors as well. For example, the timing of normal SPO1 development and assembly requires a highly ordered pattern of synthesis of DNA, enzymes, and structural proteins, which is dependent upon the integrity of the complexly sequenced SPO1 messenger RNA transcription program. A general slowdown in some or all of these steps would result in a substantial delay in intracellular phage development. The observation that no significant delay in lysis occurred if nalidixic acid was added after phage DNA synthesis was well under way may indicate that factors necessary for lysis were rapidly accumulating by that time.

It is of interest to compare our results on SPO1 with previously reported effects of nalidixic acid and mitomycin C on coliphage development. Unlike SPO1, the DNA coliphages T2r, T5, eX174, and R have been reported not to produce infectious progeny particles in the presence of nalidixic acid, although some λ induction did occur; DNA synthesis was not measured (11). A differential inhibition of DNA synthesis in uninfected and T2r-infected *E. coli* cells was observed in the presence of mitomycin C, but the phage particles produced under these conditions were noninfectious (8-10).

In contrast to these results with coliphages, nearly normal SPO1 development occurred in the presence of nalidixic acid, and viable progeny particles were produced. These properties suggest that nalidixic acid might be an extremely useful drug in facilitating studies of virus-specific DNA synthesis (certainly for SPO1, and for other phages as well). For example, it should be especially useful in studies of replication in singly infected cells.

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**LITERATURE CITED**


