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Bactericidal Action of Nalidixic Acid on *Bacillus subtilis*

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ABSTRACT

COOK, THOMAS M. (Sterling-Winthrop Research Institute, Rensselaer, N.Y.), KAREN G. BROWN, JAMES V. BOYLE, AND WILLIAM A. GOSS. Bactericidal action of nalidixic acid on *Bacillus subtilis*. J. Bacteriol. **92**:1510–1514. 1966.—Nalidixic acid at moderate concentrations exerts a bactericidal action upon the gram-positive bacterium *Bacillus subtilis*. The synthesis of deoxyribonucleic acid (DNA) in *B. subtilis* is selectively inhibited by nalidixic acid at concentrations approximating the minimal growth inhibitory concentration. Higher concentrations (25 μg/ml) result in a 30 to 35% degradation of DNA. After extended exposure to nalidixic acid, protein synthesis is also depressed. Cells of *B. subtilis* treated with nalidixic acid exhibit characteristic morphological abnormalities including cell elongation and development of gram-negative areas. From the results presented, it can be concluded that the mode of action of nalidixic acid upon susceptible bacteria is similar for both gram-positive and gram-negative species.

It has been demonstrated that the bactericidal action of the chemotherapeutic agent nalidixic acid (1,4-dihydro-1-ethyl-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid) on *Escherichia coli* is associated with a selective inhibition of deoxyribonucleic acid (DNA) synthesis (3, 4). Although generally more active against gram-negative bacteria, nalidixic acid is not without effect upon certain gram-positive species. One gram-positive organism found to be quite susceptible to nalidixic acid is *Bacillus subtilis*. This report presents the results of some preliminary studies of the action of nalidixic acid on *B. subtilis*.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. Two strains of *B. subtilis* were used: ATCC 6051 and ATCC 6633. *B. subtilis* ATCC 6051 was grown with shaking at 37 C in 50 ml of a defined medium contained in a 250-ml Erlenmeyer flask. This medium had the following composition: Na_2SO_4, 0.2 g; KCl, 0.076 g; Na_2HPO_4, 0.066 g; NaCl, 0.015 g; $MgCl_2 \cdot 6H_2O$, 0.042 g; $FeCl_3 \cdot 6H_2O$, 0.0025 g; $MnCl_2 \cdot 4H_2O$, 0.0018 g; $ZnCl_2$, 0.001 g; NH_4Cl, 0.25 g; monosodium glutamate, 0.25 g; glucose, 1.0 g; distilled water, 100 ml. This medium was supplemented with 0.5% Casamino Acids (Difco) and 25 μg/ml of DL-tryptophan for use with *B. subtilis* 6633. Trypticase Soy Broth (BBL) was used when a complex growth medium was desired. Bacterial viability was estimated by planting appropriate dilutions (in 1% peptone-water) of the cultures in Tryptone Glucose Agar (Difco).

Photomicroscopy. Smears prepared from 3-hr cultures of *B. subtilis* ATCC 6051 were stained with crystal violet and photographed with a Zeiss Ultraphot II microscope on Panatomic-X film (Eastman Kodak Co., Rochester, N.Y.).

Incorporation of radioactive precursors. Synthesis of macromolecules was measured by following the incorporation of radioactive precursors as previously described (4). Incorporation of C^{14}-labeled guanine into ribonucleic acid (RNA) and DNA was monitored by use of the modified Schmidt-Thannhauser technique of Roodyn and Mandel (5). Protein synthesis was measured by incorporation of C^{14}-labeled L-leucine into hot trichloroacetic acid-insoluble material.

Studies on the stability of RNA and DNA in the presence of nalidixic acid were carried out with *B. subtilis* 6051. The cells were incubated for five generations in basal medium supplemented with 20 μg/ml of guanine and 0.04 μc/ml of guanine-*8*-C^{14}. Cells were washed, resuspended in fresh unlabeled medium, and incubated at 37 C for 20 min to permit utilization of residual radioactivity in the metabolic pool. The labeled culture then was incubated in the presence and absence of nalidixic acid. Samples of whole culture were removed and extracted with cold 5% trichloroacetic acid (30 to 45 min). After removal of the acid-insoluble material by membrane filtration (Millipore, 0.45 μ; Millipore Filter Corp., Bedford, Mass.), the radioactivity of the acid-soluble filtrates was determined. The total radioactivity in RNA and

DNA was determined by the fractionation procedure described above. All radioactivity measurements were made with a liquid scintillation spectrometer (Packard Instrument Corp., Chicago, Ill.).

Radioisotopes. Radioactive guanine-*8-C*^{14} (17.6 mc/mmole) and L-leucine-*2-C*^{14} (7.31 mc/mmole) were purchased from Calbiochem.

RESULTS

Bactericidal action of nalidixic acid on B. subtilis. Nalidixic acid inhibited the growth of *B. subtilis* at moderate concentrations. In complex medium (Trypticase Soy Broth), the minimal growth inhibitory concentration was approximately 5 μg/ml for both *B. subtilis* ATCC 6051 and *B. subtilis* ATCC 6633.

The effect of nalidixic acid on bacterial viability was examined in shake flask cultures. A definite bactericidal action was demonstrated despite technical difficulties caused by the tendency of these organisms to form chains of cells. The most reproducible results were obtained when appropriate dilutions (in tryptone-glucose-agar, 45 C) were added to petri dishes containing 5 ml of base layer agar and subsequently overlaid with 5 ml of agar of the same composition. Under these conditions, each viable colony formed represented growth from a single cell or a chain of cells, and was counted as representative of one viable cell.

Exposure of *B. subtilis* ATCC 6051 to 10 μg/ml of nalidixic acid during growth in the synthetic medium resulted in a 90% loss in viability in 3 hr (Table 1). On the other hand, cells treated with nalidixic acid in nitrogen-deficient synthetic medium demonstrated no loss of viability (Table 2). Essentially similar results were obtained with *B. subtilis* ATCC 6633 growing in Trypticase Soy Broth (Table 3). In this case, a 50% reduction in the viable population was observed after a 2-hr exposure to 10 μg/ml of nalidixic acid.

Morphological changes in nalidixic acid-treated cells. It was noted that despite the loss of viability as shown by colony counts the turbidity of the nalidixic acid-treated cultures increased. This suggested the formation of elongated cells, as previously described with *E. coli* (3). Microscopic examination of crystal violet-stained smears from such cultures did in fact reveal the characteristic elongated cells (Fig. 1). After 3 hr of incubation with 2.5 μg/ml of nalidixic acid, the cells (Fig. 1B) were somewhat longer than cells from the untreated control culture (Fig. 1A). At higher concentrations, the cells became even longer (Fig. 1C and D). Exposure to 25 μg/ml of nalidixic acid resulted in elongated cells (Fig. 1E) which exhibited numerous unstained areas (Fig. 1F). Examination of Gram-stained smears from such cultures revealed that many of the elongated cells had developed gram-negative regions or were completely gram-negative.

Effect of nalidixic acid on biosynthesis of macro-

TABLE 1. *Bactericidal action of nalidixic acid on Bacillus subtilis ATCC 6051 in synthetic medium*

Nalidixic acid concn	Viable cells per ml	
	0 hr	3 hr
μg/ml		
0.0	1.7×10^7	1.5×10^8
2.5	1.3×10^7	3.8×10^7
5.0	1.5×10^7	4.6×10^6
10.0	1.4×10^7	1.8×10^6
25.0	1.1×10^7	1.3×10^6

TABLE 2. *Effect of nalidixic acid on nongrowing cultures of Bacillus subtilis 6051*[a]

Time of exposure	Control (complete)	Treated (10 μg/ml of nalidixic acid)	
		Complete	Deficient
min			
0	1.7×10^7[b]	1.5×10^7	1.5×10^7
30	2.3×10^7	1.1×10^7	1.3×10^7
60	4.7×10^7	4.3×10^6	1.6×10^7
90	6.8×10^7	2.1×10^6	1.3×10^7
120	9.0×10^7	1.6×10^6	1.8×10^7

[a] A culture of *B. subtilis* 6051 was depleted of its endogenous nitrogen pool by incubation at 37 C until the turbidity was constant. At zero-time, the culture was divided into three portions, two of which received NH_4Cl (0.25%) and monosodium glutamate (0.25%), and one receiving no supplements (deficient). All cultures were incubated at 37 C.

[b] Results expressed as cells per milliliter.

TABLE 3. *Bactericidal action of nalidixic acid on Bacillus subtilis ATCC 6633 in complex medium (Tripticase Soy Broth)*

Nalidixic acid concn	Viable cells per ml			
	0 hr	1 hr	1.5 hr	2 hr
μg/ml				
0	1.0×10^7	1.8×10^7	2.1×10^7	6.8×10^7
2.5	1.0×10^7	2.3×10^7	6.2×10^7	7.8×10^7
5.0	1.0×10^7	1.7×10^7	2.1×10^7	2.6×10^7
10.0	1.0×10^7	1.3×10^7	8.4×10^6	5.6×10^6
25.0	1.0×10^7	1.1×10^7	5.3×10^6	2.7×10^6

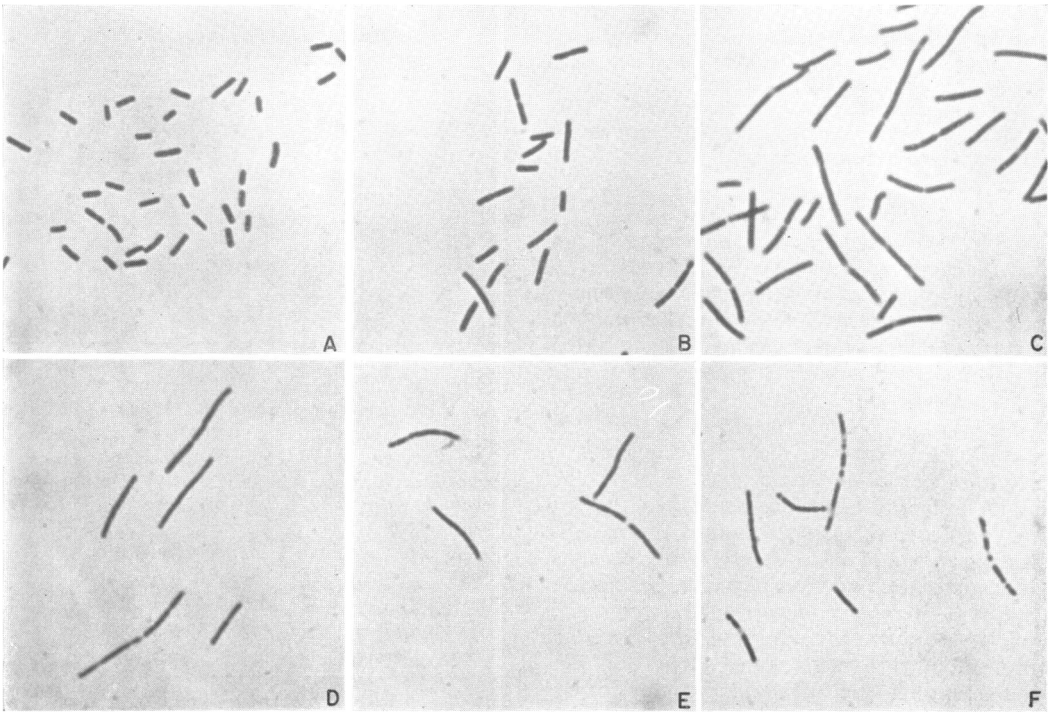

FIG. 1. *Morphological changes in cells of Bacillus subtilis ATCC 6051 after 3 hr of treatment with nalidixic acid. Smears were stained with crystal violet and photographed with a Zeiss Ultraphot II microscope at a magnification of* × *1,800. (A) Control, (B) 2.5 μg/ml, (C) 5.0 μg/ml, (D) 10 μg/ml, (E, F) 25 μg/ml.*

molecules. Nalidixic acid produced only a slight impairment of protein synthesis during the first 60 min of exposure (Table 4). However, longer treatment periods resulted in progressive inhibition of the incorporation of L-leucine-2-C^{14} into hot trichloroacetic acid-insoluble material. After a 90-min exposure to 25 μg/ml of nalidixic acid, leucine incorporation was inhibited 48%. In contrast, treatment with even low levels of nalidixic acid produced a rapid and profound inhibition of DNA (but not RNA) synthesis. Treatment with 10 μg/ml of nalidixic acid caused an immediate and complete cessation of guanine incorporation into DNA, but had no effect on incorporation into RNA. As can be seen in Fig. 2, this selective inhibition of DNA synthesis in *B. subtilis* is accompanied by a distinct reduction in the viable-cell population. The increase in turbidity observed in cultures treated with bactericidal concentrations of nalidixic acid reflects the unabated synthesis of bulk RNA and protein.

Stability of nucleic acids in the presence of nalidixic acid. Treatment of *B. subtilis* ATCC 6051 with nalidixic acid (25 μg/ml) resulted in an increase in acid-soluble material and a concomitant decrease in acid-insoluble DNA (Fig. 3). A small decrease in the radioactivity of the RNA fraction with continued incubation was observed in both the control and the nalidixic acid-treated cultures. These results indicate that the increase in acid-soluble material is due primarily to degradation of DNA. This is substantiated further by the close correlation between the quantity of radioactive material disappearing from the DNA fraction and the amount appearing in the acid-soluble fraction (Table 5). A 30 to 35% degradation of DNA occurred under these conditions.

TABLE 4. *Effect of nalidixic acid and chloramphenicol (CAP) on the incorporation of* L-*leucine-2-C^{14} by Bacillus subtilis ATCC 6051*

Time of incubation	CAP (25μg/ml)	Nalidixic acid (μg/ml)			
		5	10	15	25
min					
60	92[a]	0	0	11	20
90	96	21	35	43	48

[a] Results expressed as percentage of inhibition.

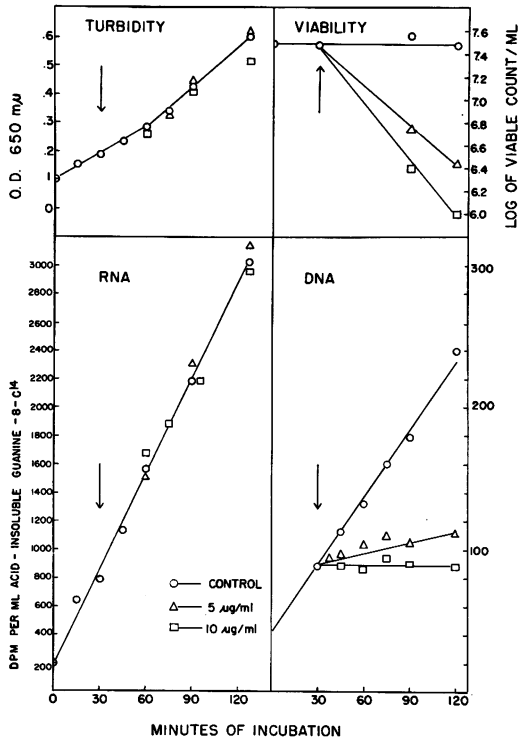

FIG. 2. *Selective inhibition of DNA synthesis in Bacillus subtilis ATCC 6051 by nalidixic acid. Cultures supplemented with 0.01 μc/ml of guanine-8-C¹⁴ were incubated for 30 min before addition of nalidixic acid (time of addition shown by the arrow). Incorporation of guanine-8-C¹⁴ into the RNA and DNA fractions was monitored by the method of Roodyn and Mandel (5).*

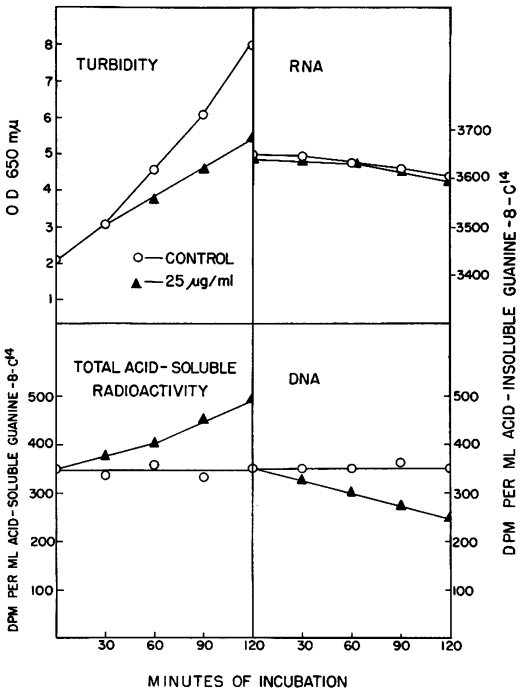

FIG. 3. *Stability of RNA and DNA of Bacillus subtilis ATCC 6051 exposed to nalidixic acid. A culture prelabeled by growth in the presence of guanine-8-C¹⁴ (0.04 μc/ml) was monitored for acid-soluble and acid-insoluble materials during growth in the presence and absence of nalidixic acid (25 μg/ml).*

TABLE 5. *DNA degradation in Bacillus subtilis ATCC 6051 exposed to nalidixic acid (25 μg/ml)*

Time of incubation	Acid-insoluble guanine-8-C¹⁴ (dpm[a]/ml)	Decrease	Acid-soluble guanine-8-C¹⁴ (dpm/ml)	Increase
min		%		%
0	352	0	352	0
30	333	6	387	10
60	313	11	400	14
90	267	21	460	30
120	240	32	480	36

[a] Disintegrations per minute.

DISCUSSION

These results demonstrate the similarities between the susceptibility of *B. subtilis* and *E. coli* to nalidixic acid, with respect to the selective inhibition of DNA synthesis, "unbalanced" metabolism, DNA degradation (in some cases), and morphological abnormalities (2, 3, 4). These findings indicate that some basic mechanism(s) associated with DNA synthesis in *B. subtilis* and *E. coli* is equally sensitive to nalidixic acid. The comparative susceptibility of this gram-positive bacterium and *E. coli* to nalidixic acid suggests that the decreased susceptibility of some gram-positive bacteria reflects differences in permeability.

Nalidixic acid has no lethal effect on nongrowing cultures of *E. coli* (3). Under the same conditions, *B. subtilis* 6051 is not susceptible to the lethal action of nalidixic acid. The degradation of DNA and loss of viability in *B. subtilis* 6051 are correlative. Since there is no detectable degradation of *E. coli* DNA under conditions of restricted protein and RNA synthesis (2), it would not be surprising to find similar results with *B. subtilis* 6051.

Our finding of selective inhibition of DNA synthesis in *B. subtilis* by nalidixic acid should permit insight into some fundamental questions regarding the process of genetic transformation. Recently, Tomasz and Mosser (6) demonstrated that inhibition of RNA or protein synthesis, or

both, blocked the establishment of the competent state, presumably by inhibiting formation of the pneumococcal activator substance. A selective inhibition of DNA synthesis by nalidixic acid, in the presence of RNA and protein synthesis, would give some insight into the ability of cells to produce activator as well as providing information on what specific metabolic activities are required for activation. If the activator substance is a proteinlike macromolecule, it would be interesting to determine the effect of DNA inhibition on its synthesis as well as the ability of cells to develop the competent state in the absence of DNA synthesis. However, to obtain completely unambiguous results, the possibility of nalidixic acid-induced DNA degradation in the *B. subtilis* transformation system would have to be eliminated.

ACKNOWLEDGMENT

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