JOURNAL OF BACTERIOLOGY, Feb., 1966
Vol. 91, No. 2

Mechanism of Action of Nalidixic Acid on *Escherichia coli*

V. Possible Mutagenic Effect

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Received for publication 25 September 1965

ABSTRACT

COOK, THOMAS M., (Sterling-Winthrop Research Institute, Rensselaer, N.Y.), WILLIAM A. GOSS, AND WILLIAM H. DEITZ. Mechanism of action of nalidixic acid on *Escherichia coli*. V. Possible mutagenic effect. J. Bacteriol. 91:780–783. 1966.—With a streptomycin-dependent organism, *Escherichia coli* ATCC 11143, it has been shown that exposure to nalidixic acid, under conditions permitting some bactericidal action, results in an increased number of streptomycin-independent bacteria among the survivors. This effect is evident only with proliferating cultures, and is related to the concentration of nalidixic acid and the duration of exposure.

The bactericidal action of nalidixic acid (1-ethyl - 1,4 - dihydro - 7 - methyl - 4 - oxo - 1,8 - naphthyridine-3-carboxylic acid) on *Escherichia coli* has been shown to involve inhibition of deoxyribonucleic acid (DNA) synthesis (6). Inasmuch as some other agents which affect DNA synthesis exert mutagenic effects, the question arises as to whether the action of nalidixic acid is accompanied by genetic changes. This report presents results which indicate a possible mutational effect of nalidixic acid on a selected strain of *E. coli*.

MATERIALS AND METHODS

Culture. *E. coli* ATCC 11143, which is the streptomycin-dependent mutant Sd-4 of Demerec, was obtained from the American Type Culture Collection. This strain of *E. coli* was originally employed by Bertani (1) for studies of mutagenesis with the streptomycin-dependence marker.

Tests for mutagenicity with nonproliferating cell suspensions. The method described by Bertani (1) for testing mutagenic effects of compounds was employed. This method involves determining the increase in the frequency of streptomycin-independent cells among the survivors after exposure of a nonproliferating suspension of streptomycin-dependent bacteria to the test agents. When samples of *E. coli* ATCC 11143 are plated on streptomycin-free agar, most of the cells divide only a few times, and colonies are not formed. Only those cells which have reverted to streptomycin-independence will give rise to colonies on streptomycin-free agar. These streptomycin-independent cells will be referred to as mutants, although they have not been characterized further.

An overnight culture in nutrient broth containing 10 μg/ml of streptomycin (as the sulfate) was harvested by centrifugation, washed in 0.85% saline, and resuspended in 0.85% saline to an optical density of 0.9 to 1.0 (600 mμ). The washed cell suspension was dispensed into sterile tubes at 5 ml per tube, and the volume was brought to 10 ml with saline and solutions of the compounds to be tested. After 60 min of incubation at 37 C, samples were taken for enumeration of viable cells and mutants. Counts of viable cells were made by plating suitable dilutions in nutrient agar containing 100 μg/ml of streptomycin. Streptomycin-independent mutants were demonstrated by pipetting 0.5-ml amounts of sample (undiluted, 1:2.5, and 1:5 dilution) to 100 ml of melted nutrient agar and pouring into large petri dishes. After 7 days of incubation at 37 C, the streptomycin-independent colonies were counted.

Tests for mutagenicity with proliferating cultures. To test compounds for mutagenic effects on proliferating cultures, the method of Bertani (1) was modified slightly. Cultures proliferating in nutrient broth with 1.0 μg/ml of streptomycin, or in a mineral salts-glucose medium (3) with 5.0 μg/ml of streptomycin, were divided into several portions. Test compounds were added at appropriate concentrations, and incubation continued at 37 C. Samples for counts of total viable cells and of streptomycin-independent mutants were taken as described above.

Chemicals. Azaserine was a gift from Parke, Davis & Co., Detroit, Mich. Nitrofurazone was a gift from Eaton Laboratories, Norwich, N.Y. Propiolactone was obtained from Eastman-Kodak, Rochester, N.Y.

RESULTS

Reversion to streptomycin independence in nonproliferating cell suspensions of E. coli ATCC

TABLE 1. *Comparison of the effects of nalidixic acid and other agents on reversion to streptomycin independence in nonproliferating cell suspensions of Escherichia coli ATCC 11143 after 1 hr of treatment*

Expt	Treatment	No. of viable cells per ml	Streptomycin-independent mutants per ml	Relative frequency*
1	Control	2.02×10^8	38	19
	β-Propiolactone (20 μg/ml)	1.03×10^8	232	225
	β-Propiolactone (50 μg/ml)	0.92×10^8	874	950
2	Control	1.1×10^8	28	25
	Nalidixic acid (10 μg/ml)	0.87×10^8	38	44
	Nalidixic acid (50 μg/ml)	0.77×10^8	44	57
	Azaserine (2.5 μg/ml)	1.5×10^8	5,280	3,520
3	Control	2.5×10^8	14	6
	Nalidixic acid (25 μg/ml)	2.9×10^8	24	8
	Nalidixic acid (50 μg/ml)	3.7×10^8	25	7
	Nitrofurazone (12 μg/ml)	1.9×10^8	20	11
	Azaserine (2.5 μg/ml)	1.1×10^8	6,500	5,900

* Number of streptomycin-independent mutants per 10^8 viable cells plated.

11143. First, we examined the action of several agents known to be mutagenic for bacteria (β-propiolactone, azaserine, and nitrofurazone). After these tests, nalidixic acid was compared with these known mutagens.

β-Propiolactone and azaserine. Under our test conditions, β-propiolactone was found to increase greatly the reversion to streptomycin independence in nonproliferating cell suspensions of *E. coli* ATCC 11143. As shown in Table 1, the effect of β-propiolactone on reversion to streptomycin independence was proportional to the concentration employed. After 1 hr of exposure to the highest concentration used (50 μg/ml), the relative frequency of streptomycin-independent mutants was increased approximately 48-fold above that of the untreated control suspension.

Azaserine was found to be a potent mutagenic agent in this system also. Treatment with azaserine (2.5 μg/ml) increased the relative frequency of streptomycin-independent mutants to a value 100 to 1,000 times that of the untreated control (Table 1).

Nitrofurazone and nalidixic acid. In contrast to the above-mentioned agents, no clear-cut effects on reversion to streptomycin independence were evident with nitrofurazone or nalidixic acid (Table 1). At concentrations of these compounds known to be inhibitory to proliferating cultures, there was little or no increase in the relative frequency of streptomycin-independent mutants after treatment of nonproliferating cell suspensions.

Reversion to streptomycin independence in cultures of E. coli ATCC 11143 proliferating in nutrient broth. Nalidixic acid has been shown to exert a bactericidal effect only upon proliferating cultures (5). It was imperative, therefore, to determine whether or not nalidixic acid would exhibit mutagenic properties under conditions permitting the lethal action of the drug. Nutrient broth cultures supplemented with a low level of streptomycin (1 μg/ml) were employed in an attempt to allow growth while minimizing carryover of streptomycin to the challenge agar. Although growth was suboptimal under these conditions, it was possible to demonstrate mutational effects of the test agents in this manner.

β-Propiolactone. It was found that β-propiolactone was mutagenic for cultures proliferating in nutrient broth as well as for nonproliferating cell suspensions. The effect of β-propiolactone concentration on the induction of streptomycin-independent mutants in nutrient broth cultures is shown in Table 2.

Nitrofurazone. Although nitrofurazone was neither bactericidal nor mutagenic for the nonproliferating cell suspensions, it exerted marked bactericidal and mutational effects upon the proliferating cultures in nutrient broth. Both effects were dose-dependent, as can be seen from the results summarized in Table 2. The absolute number, as well as the relative frequency, of streptomycin-independent mutants present in the culture was increased by treatment with nitrofurazone. After a 1-hr exposure to the highest concentration tested (3 μg/ml), the viable

TABLE 2. *Induction of streptomycin-independent mutants in a proliferating nutrient broth culture of Escherichia coli ATCC 11143 by a 1-hr exposure to nitrofurazone or β-propiolactone*

Expt	Concn	No. of viable cells per ml	Streptomycin-independent mutants per ml	Relative frequency*
	μg/ml			
1. Nitrofurazone	None (zero min)	4.2×10^8	76	18
	None	5.5×10^8	54	10
	0.75	2.8×10^8	92	33
	1.50	8.3×10^7	126	154
	3.00	5.9×10^7	112	187
2. β-Propiolactone	None (zero min)	5.5×10^8	110	20
	None	6.2×10^8	90	15
	25	6.2×10^8	998	161
	50	5.5×10^8	1,756	329
	100	3.8×10^8	2,760	726

* Number of streptomycin-independent mutants per 10^8 viable cells plated.

TABLE 3. *Reversion to streptomycin independence after 1-hr treatment of proliferating nutrient broth cultures of Escherichia coli ATCC 11143 with nalidixic acid*

Concn of nalidixic acid	No. of viable cells per ml	Streptomycin-independent mutants per ml	Relative frequency*
μg/ml			
None (zero min)	4.5×10^8	40	9
None	12.6×10^8	45	4
1.0	9.3×10^8	49	5
2.5	10.6×10^8	49	5
5.0	5.4×10^8	120	22
10.0	4.9×10^8	625	128
25.0	2.9×10^8	335	116

* Number of streptomycin-independent mutants per 10^8 viable cells plated.

cell population had declined sevenfold, whereas the relative frequency of streptomycin-independent mutants increased 10-fold.

Nalidixic acid. It was found that reversion to streptomycin independence was definitely increased when nalidixic acid was added to proliferating nutrient broth cultures of *E. coli* ATCC 11143. The effect of the concentration of nalidixic acid on the induction of streptomycin-independent variants is shown in Table 3. An exposure for 1 hr to concentrations of 1.0 μg/ml and 2.5 μg/ml had no apparent effect, but higher levels of 5.0 to 25.0 μg/ml caused a significant increase in the number of streptomycin-independent organisms per milliliter, as well as their relative frequency.

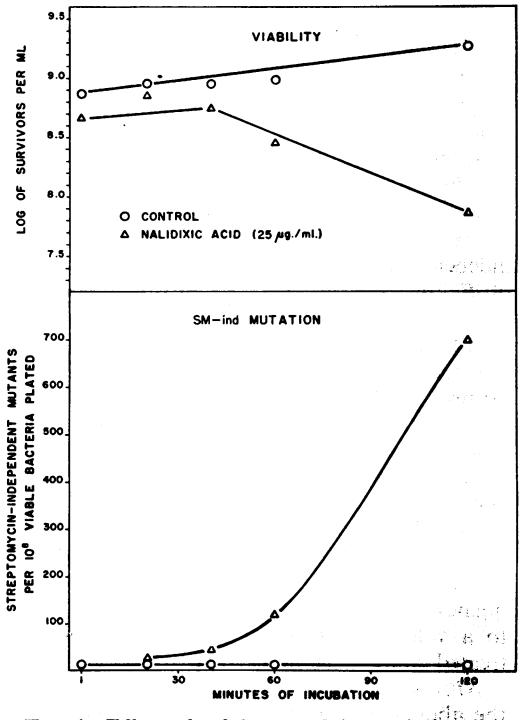

FIG. 1. *Effect of nalidixic acid (25 μg/ml) on reversion to streptomycin independence in a proliferating nutrient broth culture of Escherichia coli ATCC 11143.*

The induction of streptomycin independence in *E. coli* ATCC 11143 as a function of the dura-

tion of exposure to nalidixic acid (25 μg/ml) is illustrated in Fig. 1. It is apparent that the decline in the viable cell population is paralleled by an increase in the relative frequency of streptomycin-independent mutants.

Results comparable to those with nutrient broth were obtained also in a similar series of experiments with a mineral salts-glucose medium (3). The absolute numbers, as well as the relative frequency of streptomycin-independent mutants, was increased after treatment with nalidixic acid. This increase was related to the concentration of nalidixic acid employed and the duration of exposure.

DISCUSSION

The streptomycin-dependence system for evaluation of mutagenic agents has been thoroughly described by Bertani (1) and Demerec, Bertani, and Flint (4). Using this genetic marker, we were able to demonstrate the mutagenic properties of several known mutagenic agents: azaserine, β-propiolactone, and nitrofurazone. In each case, the mutational response was dose-dependent and represented an absolute increase in the number of mutants per milliliter of culture.

The results obtained with nalidixic acid were not surprising, considering its known effects on bacteria (5, 6). It has been shown that nalidixic acid is bactericidal only for proliferating cells. There is no loss of viability in the presence of nalidixic acid when growth is prevented by low temperatures or nutritional deficiency (5). Consequently, it was not unexpected to find that nalidixic acid enhanced reversion to streptomycin independence only in proliferating cultures.

These results are suggestive of a mutagenic action of nalidixic acid upon proliferating cultures of susceptible bacteria. To substantiate this view, it would be necessary to ascertain the effects of nalidixic acid on the relative proliferation and death rates of the dependent and independent variants. Without such information, one cannot adequately distinguish a mutagenic effect from a small, but finite, selective effect. Considering the complexities of the streptomycin-independence system, it may be more advantageous to resolve this point with another genetic marker. Despite these uncertainties, it appears likely to us that the effect of nalidixic acid on reversion to streptomycin independence is a consequence of the same series of events which results in the death of the organism. Synthesis of DNA is halted by nalidixic acid by a mechanism as yet unknown. Subsequently, the DNA of the cell, in at least some strains of *E. coli*, is degraded to acid-soluble products (2). It is possible that a mutational event is connected with miscoding during resumption of DNA synthesis after removal of the drug or during repair of nuclease damage.

ACKNOWLEDGMENT

We acknowledge the technical assistance of John G. Babonis in these studies.

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