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Nalidixic Acid for Enrichment of Auxotrophs in Cultures of *Salmonella typhimurium*

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Nalidixic acid kills only growing cells of *Salmonella typhimurium* and can be used to enrich for auxotrophs in populations where prototrophs predominate.

A method using penicillin to enrich for biochemically deficient mutants of bacteria in populations where prototrophs predominate was devised over two decades ago (4, 5, 12), and has since become a standard procedure (10). This technique is based on the fact that penicillin is bactericidal apparently only to growing organisms. Thus, auxotrophic mutants selectively survive exposure to penicillin in unsupplemented minimal medium. Dihydrostreptomycin (11) has also been used in a similar manner.

Nalidixic acid is another antibacterial agent that kills only growing cells (7). Here we report that, based on results obtained with *Salmonella typhimurium*, nalidixic acid (Nal) can be used successfully to facilitate selection of auxotrophs.

MATERIALS AND METHODS

Wild-type *S. typhimurium* LT2, and a deletion mutant of the wild-type strain, *hisBH22* (9), were used in these studies. The *hisBH22* strain multiplies at a rate comparable to the wild type in minimal medium supplemented with histidine. Minimal medium was the E medium of Vogel and Bonner (15) to which 0.2% glucose was added. Where appropriate, this medium was supplemented with 20 μg of histidine per ml (histidine-minimal medium). Complete medium was nutrient broth (Difco) plus 0.5% NaCl. Enriched minimal medium was minimal medium supplemented with 1% (vol/vol) of nutrient broth. On this medium, prototrophic cells form large colonies, whereas auxotrophic cells grow only to a limited extent and form small colonies. Nal was used at a final concentration of 20 μg/ml.

RESULTS

Initially, the effect of Nal on growing and nongrowing cultures of *hisBH22* was determined. *HisBH22* was incubated in minimal medium or in histidine-minimal medium, and Nal was added at 2 h after the start of incubation. In histidine-minimal medium at the time of addition of Nal, the cells were growing logarithmically with a doubling time of 59 min (Fig. 1). During the first 3 h after Nal addition a sharp decrease in viability of the culture ensued with a maximal kill time of 21 min and a total 99.8% reduction in viable cell count. Thereafter, the rate of killing became much reduced. These results are similar to those obtained when growing cultures of *Bacillus subtilis* (13) and *Escherichia coli* (6) are exposed to Nal. To test whether this markedly reduced rate of killing might be due to the accumulation of Nal-resistant mutants, or to cells which have an additional growth requirement besides histidine, survivor clones after at least 6 h of exposure to Nal were replicated to nutrient agar containing Nal and to histidine-minimal agar. Of approximately 100 clones tested for sensitivity or resistance to Nal, all were sensitive. Of approximately 200 survivor clones tested for growth on histidine-minimal medium, all grew. The small fraction of cells which survive in the presence of Nal might be very slow growing cells as suggested by Deitz et al. (6).

When *hisBH22* cells were cultured under the same conditions, but with histidine omitted from the medium to prevent growth, addition of Nal did not result in loss of viability (Fig. 1).

We next tested the ability of Nal to select for a small fraction of auxotrophs growing in mixed culture with prototrophic wild-type cells. Inocula cultures of the wild-type strain and of *hisBH22* were grown separately in minimal and in histidine-minimal medium, respectively, without agitation at 35 C. The two cultures were diluted into minimal medium containing Nal to give a final concentration of 1.9×10^7 wild-type cells per ml and 5.1×10^5 mutant cells per ml. The mixed population was incubated with aeration at 37 C for 5 h and then titered on histidine-minimal agar. One hundred twenty-six survivor clones were replicated to minimal and to histidine-minimal agar in order

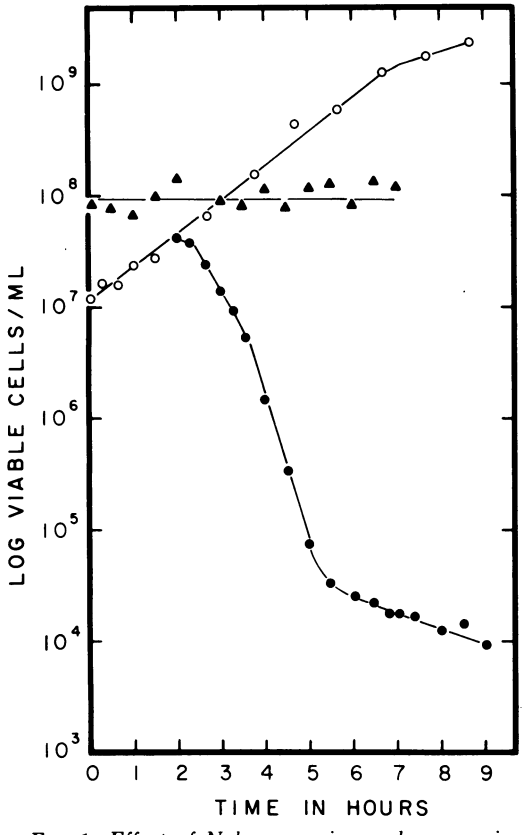

FIG. 1. *Effect of Nal on growing and nongrowing cultures of hisBH22. Growing culture: hisBH22 was grown in histidine-minimal medium at 35 C without agitation overnight, diluted into the same medium, and at time 0 the culture was incubated with aeration at 37 C. At 2 h, half of the culture was removed to a flask containing Nal (●, Nal added; ○, control without Nal). Nongrowing culture: hisBH22 was cultured as described above. The cells were pelleted by centrifugation, resuspended in minimal medium, and at time 0 the culture was incubated with aeration at 37 C (▲). At 2 h Nal was added. Viable cells were determined by plating on nutrient agar.*

to determine the fraction which were of the mutant phenotype. The total number of surviving cells was 4.0×10^5 per ml and of these 79% were *hisBH22*. Based on these data there was a 0.4% survival of the wild type and a 63% survival of the mutant.

Finally we tested the ability of Nal to select for auxotrophs induced by mutagenesis. Employing two different mutagens, ultraviolet light (UV) and diethyl sulfate, we found that Nal did selectively enrich for auxotrophic mutants and that the enriched populations yielded a variety of such mutants. Data from a UV mutagenesis experiment, which represent our current refinement of the technique, are shown in Table 1. In this experiment wild-type cells were cultured through two passages in minimal medium to eliminate any spontaneously induced auxotrophic mutants that might be present in the stock culture. A 5.0-ml portion from the second passage culture (1.5×10^9 cells per ml) was irradiated with UV light (400 ergs/mm^2) from a germicidal lamp (General Electric, G15T8) to 0.02% survival as determined by immediately plating on enriched minimal agar. The irradiated sample was diluted 1:10 in nutrient broth and incubated with aeration at 37 C for 7 h and stored at 4 C. The next day the cells were washed and resuspended in an equal volume of minimal medium, titered on enriched minimal agar, and diluted 1:10 into fresh minimal medium to a concentration of about 10^7 cells per ml. The culture was incubated with aeration at 37 C for 1 h to allow for residual growth of auxotrophic mutants. Nal was then added, and the culture was incubated for an additional 5 h. At 3 and 5 h after addition of Nal, the culture was titered on enriched minimal plates. Small clones present after 2 days of incubation were transferred to a nutrient agar plate, and this master plate was replicated to a minimal agar to assess auxotrophy, and then to an auxanographic series of amino acid pool plates designed to detect single amino acid requirements (10). The exposure to Nal, which led to a 97% reduction in total viability, resulted in a greater than 10-fold increase in auxotrophs (Table 1). Of the 15 auxotrophs obtained, eight showed a requirement for a particular amino acid, either isoleucine, tyrosine, tryptophan, phenylalanine, methionine, or arginine. The auxotrophic requirements of the seven other mutants were not determined.

DISCUSSION

The Nal selection procedure described here for *S. typhimurium* should be adaptable to other species of Nal-sensitive bacteria and of-

TABLE 1. *Nal enrichment of auxotrophic mutants in an UV-treated culture of S. typhimurium LT2*

Incubation time[a] (h)	Viability		Mutant analysis		
	Survivors (cells/ml)	Surviving fraction (%)	Total colonies tested	Mutant colonies	Mutant fraction (%)
0	9.6×10^6		389	0	<0.26
4	3.8×10^6	40	377	1	0.27
6	2.6×10^5	3	566	14	2.5

[a] Nal was added at 1 h.

fers an alternate method for auxotrophic enrichment in strains or species resistant to penicillin. In the experiments reported here, the percentage of survival after a given time was found to vary. The rate of killing may have been influenced by the state of growth of the culture at the time that Nal was added or by prior cultural conditions. We would expect the Nal selection procedure to be most effective when, at the time of addition of Nal, prototrophs were growing at an optimal rate (6) and auxotrophs had ceased residual growth.

Generally, the primary action of Nal is to inhibit the synthesis of deoxyribonucleic acid (8) and in common with some other agents which inhibit deoxyribonucleic acid synthesis, Nal can induce prophage (14). One other potential complication in the use of Nal to select auxotrophs is the possibility that Nal itself is mutagenic (3). We have observed no apparent mutagenic effect by Nal in the pour plate procedure of Ames (1) which employs a series of *his* mutants of *S. typhimurium*. Since the various effects of Nal seem to be exerted exclusively on growing cells (2, 6), it is unlikely that nonmultiplying auxotrophic cells would be susceptible to mutation by Nal in any case. However, until more is known about the mechanism of action of Nal, the possibility of mutagenic potential should be weighed in considering its use.

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