Dependence of the Selection of Thymineless Mutants on the Acriflavine Sensitivity in *Escherichia coli* Treated with Aminopterin

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INTRODUCTION

Thymineless (*thy*⁻) mutants are obtained at a high frequency when bacteria are grown in the presence of aminopterin (APT), a folic acid antagonist (Okada, Yanagisawa & Ryan, 1960). On the other hand, the acriflavine (AF) sensitivity of *Escherichia coli* K-12 is determined by a gene *acrA* located near *lac* (Nakamura, 1965, 1968). This report shows that the frequency of *thy*⁻ mutants in *E. coli* treated with APT is dependent upon the allelism of the *acrA* gene.

METHODS

Bacterial strains. Strain N43 is an *acrA* (AF sensitive) mutant of *Escherichia coli* K-12. Strain N90 is an *acrA*⁺ (AF-resistant) recombinant from a cross W1895 Hfr *acr*⁺ × N43 F⁻ *acrA* interrupted at 4 min. Thus, most of the chromosome of N90 is common to that of N43.

Media. Broth PGY contained (per l.): polypeptone (Daigo-eiyo), 5 g.; yeast extract (Daigo-eiyo), 1 g.; NaCl, 3 g.; glucose, 1 g. Initial pH of the medium was adjusted to 7.4 with 1N-NaOH. When yeast extract was omitted it is referred to as PG. Minimal medium S corresponds to S₁-glucose of Nakamura (1965). In some cases, S was enriched with 0.25 % (w/v) Casamino acids and 20 µg./ml. of tryptophane.

Isolation of AF-resistant mutants of N43. Method 1: Since N43 is also sensitive to phenethyl alcohol, phenethyl alcohol-resistant mutants were isolated by the method of Nakamura (1968). These mutants were also resistant to AF. Method 2: AF-r mutants were directly selected on PGY agar containing AF (100 µg./ml.).

Determination of the frequency of *thy*⁻ mutants after APT treatment. S medium-grown cells were inoculated to S medium containing 500 µg./ml. APT with an inoculum size about 10⁶ cells/ml. and after 24 h. shaking, 200 µg./ml. of thymidine was added to allow growth of *thy*⁻ cells. From inoculation to full turbidity of the cultures required 117 h. for N43 and 52 h. for N90. The final cultures were streaked on PG agar supplemented with 10 µg./ml. thymidine and clones were isolated. Thymine requirement of the clones was confirmed on solid and liquid S media with and without thymidine enrichment (10 µg./ml.).

RESULTS

Frequency of *thy*⁻ mutants. Table 1 shows the frequency of the *thy*⁻ mutants in the AF-s strain N43 and the AF-r strain N90 treated with APT. The N43 cultures were much richer in mutants than the N90 cultures. When small colonies were selectively picked from the APT-treated N90 PG plate, the frequency of *thy*⁻ mutants was somewhat higher than when

large colonies were picked. With N43, on the other hand, no such relationship between colony size and frequency of *thy*⁻ mutants was obtained.

When small and large colonies from the APT-treated N90 plates were picked and sub-cultured separately in PGY medium, colony size was not clonal. No significant difference in AF sensitivity between clones of the two types of colonies was detected when they were tested by the gradient plate method (Nakamura, 1965). Thus, the colony size effect on the yield of *thy*⁻ mutants may be due to a partial shortage of exogenous thymidine.

Table 1. Frequency of thymineless mutants in strains N43, AF-s and N90, AF-r, treated with APT*

Strain	Expt. no.	No. of clones tested	Frequency (%) of <i>thy</i> ⁻ mutants
N43	1	200	60.5
	2	100	98.0
	3	90	74.4
	4	100	93.0
	5	100	71.0
	6†	100	100
	7‡	100	98.0
N90	1	200	0.5
	2	168	0
	3†	100	5.0
	4†	100	8.0
	5†	200	8.0
	6†	100	8.0
	7‡	100	0
	8‡	100	0
	9‡	100	0

* Cultures treated with aminopterin (500 µg./ml.) were then supplemented with thymidine (200 µg./ml.) and spread on PG agar medium supplemented with thymidine (10 µg./ml.). After overnight incubation colonies were tested for thymine requirement.

† and ‡ Small and large colonies, respectively, were selectively picked and tested for thymine requirement.

Mutants of N43 having variable levels of AF resistance were obtained by seeding N43 on a gradient plate containing AF (100 µg./ml.) in the upper layer and picking colonies which appeared along the AF concentration gradient. Clones of these mutants were subjected to the APT treatment and the yield of *thy*⁻ mutants was determined. The frequency of *thy*⁻ mutants correlated positively with the level of AF sensitivity. Therefore, there is little doubt that the yield of *thy*⁻ mutants after APT treatment is affected by the *acrA* gene.

Cross-sensitivity between AF and APT. Overnight cultures of strains N43 and N90 were inoculated into Casamino acids-enriched S medium containing 40, 100, and 200 µg./ml. APT and shaken at 37°. At intervals samples were plated on PGY agar to count viable organisms. With N43, growth was remarkably inhibited (about 50 % of control rate) by 100 µg./ml. and thymineless death occurred after one generation in 200 µg./ml. APT. With N90, on the other hand, growth was not inhibited by 100 µg./ml. at all and only slightly inhibited (about 85 % of control rate) by 200 µg./ml. APT.

Three revertants of N43 selected for resistance to phenethyl alcohol and one, N562 (Nakamura, 1968), selected for resistance to AF were tested for sensitivity to APT. All the revertants were more resistant to APT than their parents. Therefore, it is concluded that gene *acrA* determines sensitivity to APT.

DISCUSSION

In *acrA*⁻ cultures *thy*⁻ mutants possess much more selective advantage in the presence of APT than *thy*⁻ mutants in *acrA*⁺ cultures. This is because growth of the *acrA*⁻ strain is inhibited more strongly by APT than that of the *acrA*⁺ strain and *thy*⁻ mutants are resistant to APT, which therefore acts as a selective agent, not as a mutagen (Okada *et al.* 1960).

APT binds strongly to the enzyme dihydrofolate reductase and inhibits production of tetrahydrofolate and eventually, thymidylate (Jukes & Broquist, 1963). Several explanations have been proposed to account for alterations of APT sensitivity in bacteria. A change in dihydrofolate reductase that increases its activity or makes the enzyme less sensitive to APT (Sirotnak, Donati & Hutchison, 1964*a, b*; Albrecht, Palmer & Hutchison, 1966); formation of a metabolic bypass which is insensitive to APT (Wilson, Farmer & Rothman, 1966); a mutational loss of thymidylate synthetase activity (Bertino & Stacey, 1966); a change in permeability to APT. It is known that the uptake of folate and APT are essentially nil in *Escherichia coli* and some other organisms. However, strains can be selected which show an increased uptake of APT and folate and thus sensitivity to APT (Wood & Hitchings, 1959; Pine, 1960; Hitchings & Burchall, 1965). APT transport is assumed to be due to a permease specific for folate. On the other hand a general increase in permeability to various substances cannot be ruled out.

Studies on the AF sensitivity have demonstrated that *acrA*⁻ is a membrane mutation causing increased permeability to basic dyes and thus increased sensitivity to dyes and to lipophilic substances (Nakamura, 1965, 1966, 1967*a, b*, 1968). Thus *acrA*⁻ cells are more permeable to APT than wild-type cells and APT selects more strongly against *thy*⁺ *acrA*⁻ than against *thy*⁺ *acrA*⁺ and favours the growth of *thy*⁻ *acrA*⁻ mutants which are resistant to APT.

Stacey & Simon (1965) found that selection of *thy*⁻ mutant was much easier if trimethoprim, a dihydrofolate reductase inhibitor, was used and suggested that this is because *Escherichia coli* are permeable to the drug. (This is another example in *E. coli* that the efficiency of a drug for the selection of *thy*⁻ mutants depends on the cellular permeability.)

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