JOURNAL OF BACTERIOLOGY, Aug., 1965
Vol. 90, No. 2

Printed in U.S.A.

Improved Method for the Isolation of Thymine-Requiring Mutants of *Escherichia coli*

K. A. STACEY[1] AND EVA SIMSON
Department of Radiology, Yale University School of Medicine, New Haven, Connecticut

Received for publication 20 March 1965

Thymine-requiring mutants of *Escherichia coli* were rarely found before the observation by Okada, Yanagisawa, and Ryan (Z. Vererbungslehre **92**:403, 1961) that cultures of bacteria grown in the presence of high concentrations of aminopterin, thymine, purines, and serine contain a surprising number of cells unable to synthesize their own supply of thymine. They showed that these thymine-dependent mutants could grow much faster in the presence of aminopterin than the parent strains, even though both were supplied with adequate amounts of those metabolites whose syntheses are blocked by aminopterin. These observations provided the basis for a widely used method of isolation of this special class of mutants. The greater efficiency of a simplified medium was reported by Okada, Homma, and Sonohara (J. Bacteriol. **84**:602, 1962). We also found that the yield of *thy*$^-$ mutants was greatly increased if a completely minimal medium, usually M9, containing only thymine (50 μg/ml) in addition to aminopterin ($\sim$ 300 μg/ml) was used as the isolation medium. The absence of the other metabolites, for whose synthesis tetrahydrofolate is required, appears to increase the selection pressure in favor of the thymine-dependent mutants. In some experiments, after 48 hr of growth in this medium, the percentage of colony-formers unable to make their own supply of thymine approached 100%.

In these experiments, it was much more difficult to isolate *thy*$^-$ mutants from strains that require vitamin B_1 (thiamine). Pine (J. Bacteriol. **79**:827, 1960) presented evidence that aminopterin enters the cells of *Bacillus subtilis* mainly by active transport by the thiamine permease, and that the addition of small amounts of thiamine or a similar pyrimidine affords a great deal of protection against the growth inhibition caused by aminopterin (Pine, J. Bacteriol. **79**:835, 1960). It is likely that the added B_1 in the medium similarly protects the B_1-requiring strains of *E. coli*. The high concentrations of aminopterin required to inhibit *E. coli* are necessary because, like *B. subtilis*, this species lacks a permease for folic acid (Wood and Hitchings, J. Biol. Chem. **234**:2581, 1959).

The effects of other inhibitors of the enzyme dihydrofolate reductase were, therefore, investigated. We sought to establish the role of this enzyme in this method of selection, and also to find an agent which would enter the bacterial cell more readily. The two diaminopyrimidines, pyrimethamine [2,4-diamino-5-(4 chlorophenyl)-6-ethyl-pyrimidine] and trimethoprim [2,4-diamino-5-(3′4′5′-trimethoxy)-benzyl pyrimidine] (Roth, Falco, and Hitchings, J. Med. Pharm. Chem. **5**:1103, 1962), were suggested to us. The isolation of a mutant of *Lactobacillus casei*, with an absolute requirement for thymidine, which used pyrimethamine had already been reported (Singer, Elion, and Hitchings, Bacteriol. Proc., p. 127, 1958). For the *E. coli* strains we studied, however, 150 μg/ml of pyrimethamine did not efficiently inhibit bacterial growth in the presence of thymine, nor was there any preferential growth of thymine-requiring mutants. Trimethoprim, on the other hand, proved a very convenient agent, even with the B_1-dependent strains that had been refractory in the aminopterin method. This is consistent with the measurements by Burchall and Hitchings (Intern. Congr. Biochem, 6th, vol. 5, p. 248, 1964) of the capacity of these drugs to inhibit the *E. coli* dihydrofolate reductase. The inhibition constant of trimethoprim is 10,000-fold greater than that of pyrimethamine; indeed, it is comparable with that of aminopterin, with the added virtue of being able to enter the cell freely. For the *E. coli* strains studied so far, growth in M9 containing 5 to 10 μg/ml of trimethoprim was slow and usually yielded filamentous cells. Upon the addition of 50 μg/ml of thymine, cell concentrations of more than 10^8 bacteria per milliliter were reached in 48 to 72 hr, and a large fraction, often more than half, of the colony-forming cells were thymine-dependent.

All of the mutants we have so far examined in detail are resistant to more than 100 μg/ml of trimethoprim, and grow with nearly normal doubling times in the presence of this amount of

[1] On leave from the Microbial Genetics Research Unit, Medical Research Council, Hammersmith Hospital, London, England.

the antimetabolite. As with aminopterin, the selection depends on the relative absence of growth inhibition of the mutant compared with the parent strain. In minimal media, they all require more than 40 μg/ml of thymine, as has generally been the case for the mutants isolated by use of aminopterin. A further mutation is necessary before these strains will grow on low concentrations (1 μg/ml) of thymine (Pritchard and Stacey, *unpublished data*). The mechanism of selection of *thy*$^-$ mutants and the characteristics of the dihydrofolate reductase in these mutants will be the subject of another paper. G. H. Hitchings (*personal communication*) has made similar observations with a dimethoxy-analogue of trimethoprim.

We thank K. F. Nakken of Norsk Hydro's Institute for Cancer Research, Oslo, Norway, and G. Bertino, G. Fisher, and P. Howard-Flanders of Yale University Medical School for their help and comment. We also thank G. H. Hitchings of Burroughs, Wellcome and Co., Inc., Tuckahoe, N.Y., for the gift of trimethoprim.

This investigation was supported by Public Health Service grants CA-06519 and GM-11074.