A New Effective Method for Obtaining Adenine-requiring Mutants of *Bacillus subtilis*

Sir:

The isolation of auxotrophic mutants can be facilitated by enrichment of the population of auxotrophic mutants. For this purpose, usually the penicillin method¹⁾ is used, and its several improved methods^{2,3)} were reported. By these methods, it is yet very difficult or somewhat complicated to increase the proportion of the auxotrophic mutant. V Iyer's heat shock method⁴⁾ is very efficient one, but its application is limited to spore-forming bacteria. T. Okada et al. reported the aminopterin method⁵⁾ specific for obtaining thymine-requiring mutant of *E. coli* and *S. typhimurium*, and they said that thymine-requiring mutant grew faster than the parent strain in the medium supplemented with thymine and aminopterin. Aminopterin is a folic acid analogue and is known to interfere with the reaction in the de novo synthesis of purine ribotide GAR→FGAR and AICAR→FAICAR as well as the reaction of uridylic acid to thymidylic acid. So we considered the aminopterin method, which had been thought to be specific for obtaining thymine-requiring mutant, would be available for obtaining adenine-requiring mutants. After

investigation with several modification of T. Okada's method, aminopterin turned out to be very effective agent for enrichment of population of the adenine-requiring mutants of *Bacillus subtilis*.

The procedure is as follows:

The strain used was *Bacillus subtilis*. Five ml of minimum medium (NH₄Cl 0.5%, (NH₄)₂CO₃ 0.5%, K₂HPO₄ 0.6%, KH₂PO₄ 0.2%, MgSO₄·7 H₂O 0.01%, glucose 0.1%, polypeptone 0.03%, tap water) was inoculated with 0.1~0.02 ml of culture on the same medium for 18 hrs. and supplemented with 0.5 ml of aminopterin (1 mg/ml.). After shaking culture at 37°C for 24 hrs., 1 ml of adenine sulfate (2 mg/ml) was added, incubation continued for 24~48 hrs., and the cells reached full growth. Then the cultured medium was stored at -20°C overnight. After suitable dilution, it was plated on supplemented agar medium (MM+adenine), and adenine-requiring mutants were obtained by the replica-plating technique.

The storage in the cold resulted in 1.0% survival with marked enrichment of the proportion of the adenine-requiring mutants. We don't know the reason why the proportion o

TABLE I. THE REQUIREMENT OF THE ADENINE-REQUIRING MUTANTS OBTAINED BY THE AMINOPTERINE METHOD

Supplement to the medium	AICA	Hypoxanthine	Adenine	Guanine	Thymine	Numbers of mutants
a	—	+	+	+	—	9
b	—	—	+	—	—	134
+ Growth — No growth						
AICA: 5-amino-4-imidazolecarboxamide						

1) Bernard D. Davis, *J. Am. Chem. Soc.*, **70**, 4267 (1948).

2) Martin Lubin, *J. Bact.*, **83**, 696 (1962).

3) Luge Gorini, *Science*, **131**, 609 (1960).

4) V. Iyer, *J. Bact.*, **79**, 309 (1960).

5) T. Okada, J. Homma and H. Sonohara, *ibid.*, **64**, 602 (1962).

the adenine-requiring mutants became rich by the cold treatment.

The fractions of the auxotrophic mutants obtained by the method above mentioned were as follows

18/43, 23/88, 27/36, 13/28, 26/45, 36/64.

When the cold treatment was not given, the fractions of the auxotrophic mutants were approximately 1%.

The requirements of the auxotrophic mutants obtained are shown in Table I.

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