ENRICHMENT OF AUXOTROPHIC MUTANT POPULATIONS BY RECYCLING

MARTIN LUBIN

Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

Received for publication October 2, 1961

The isolation of auxotrophic mutants can often be facilitated by a recycling procedure. Two applications are discussed here, the first involving selection of mutants of *Escherichia coli* by penicillin, and the second, selection of mutants of *Serratia marcescens* by tritiated leucine.

Except for recycling, the general method of selection by penicillin (Davis, J. Am. Chem. Soc. **70**:4267, 1948; Lederberg and Zinder, J. Am. Chem. Soc. **70**:4267, 1948) was nearly the same as the modification described by Gorini and Kaufman (Science **131**:604, 1959). In an experiment designed to select for mutants defective in potassium transport, six clones of *E. coli* W were separately grown in medium A (Davis and Mingioli, J. Bacteriol **60**:17, 1950), irradiated with ultraviolet light, incubated overnight, and washed with a potassium-free medium (Sodium-A; Lubin and Kessel, Biochem. Biophys. Research Communs. **2**:249, 1960). For each culture, about 2×10^8 cells were inoculated into 30 ml of Sodium-A medium. After 1 hr of shaking at 37 C, sodium penicillin was added to a final concentration of 1000 units/ml, and 2 hr later cells were collected on Millipore filters (HA, 0.45-μ), with brief washing. The filter with adhering cells was transferred to fresh medium A with amino acids, and the culture grown overnight. The next day, a sample was taken from this, and the same procedure of treatment with penicillin and growth of survivors followed. This was continued for a total of four cycles. Mutants requiring high potassium levels for growth were looked for by replica plating (Lederberg and Lederberg, J. Bacteriol. **63**:399, 1952), but none was found after one cycle. After four cycles, however, the fractions of mutants in the six populations were 0/135, 0/177, 68/101, 2/52, 25/49, and 28/44. In other experiments, sufficient recycling usually resulted in sharp division of cell populations into two classes, one containing no detectable mutants, the other with over 50% mutants.

To isolate auxotrophic mutants of a penicillin-insensitive strain of *S. marcescens*, a culture was irradiated and grown in medium S (Labrum and Bunting, J. Bacteriol. **65**:394, 1953) enriched with yeast extract and casein hydrolysate. About 5×10^7 washed cells were placed in 1 ml of minimal medium S and incubated with shaking at 30 C for 1 hr. Tritiated DL-leucine (3 c/mmole) was then added to a final concentration of 4 μg/ml; incubation was continued for 4 more hr, at which time the tube was stored at 4 C. By 3 days, viability of the treated culture had decreased 100-fold, and the fraction of auxotrophs measured in a subinoculated culture was about 0.5%. A second cycle with tritiated leucine enriched the fraction of auxotrophs, which included many varieties, to over 10% of the surviving population.

In a previous paper (Lubin, Science **129**:839, 1959), the general method of isolation of auxotrophs by the use of highly radioactive compounds was illustrated in an experiment with tritiated thymidine. At equivalent levels in the medium, tritiated leucine turns out to be much more effective, presumably because the leucine content of the cell is much greater than the thymidine content, and possibly because of inefficient incorporation of exogenous thymidine into the deoxyribonucleic acid (Rachmeler et al., Biochim. et Biophys. Acta **49**:222, 1961). In experiments with *E. coli*, at cell densities not over 5×10^7 per ml, and tritiated leucine in the medium as above, growth for almost two generations followed by storage in the cold resulted in 0.1% survival in 24 to 48 hr, with marked enrichment of the fraction of auxotrophic mutants. Dilution of the tritiated leucine by carrier caused a marked decrease in the rate of killing.

In the penicillin recycling experiments, for unknown reasons, cultures sometimes failed to grow on transfer of the cells on Millipore filters to fresh growth medium. However, if a very small amount (0.01%) of yeast extract and casein hydrolyzate were added, cultures always grew

out from the filters, and this addition is now made routinely. Specific auxotrophs for many of the common amino acids have been selected with great ease by two or three cycles of penicillin treatment, even without irradiation. On the other hand, mutants of *Aerobacter aerogenes* defective in potassium transport were found only after eight cycles.

The explanation of the occasional requirement for many cycles of penicillin treatment is not clear. By calculation, if wild-type cells were selectively killed by penicillin, with a survival of 10^{-4}, a single mutant in a population of 10^8 cells should appear after only two cycles of treatment. For a variety of reasons (such as loss of some mutants during penicillin treatment or failure of mutants to grow as rapidly as wild-type cells in supplemented medium), the efficiency of enrichment may be imperfect and more cycles will be required. Multiple recycling also increases the probability of recovering mutants which arise spontaneously.

I am indebted to Dorothy Lubin, Joyce S. Marvel, and Abigail A. Barnes for laboratory assistance, to B. D. Davis and L. Gorini for comments, and to the U. S. Public Health Service (GSF 15-083 and RG-6712) and the National Science Foundation (G 12978) for support.

LYSIS OF LACTIC ACID BACTERIA BY LYSOZYME AND ETHYLENEDIAMINETETRAACETIC ACID

W. C. BROWN, W. E. SANDINE, AND P. R. ELLIKER

Department of Microbiology, Oregon State University, Corvallis, Oregon

Received for publication October 5, 1961

Lysis of streptococci by the enzyme actinomycetin obtained from *Streptomyces albus* (Maxted, Lancet **2**:255, 1958; McCarthy, J. Exptl. Med. **96**:555, 1952; Slade and Slamp, J. Bacteriol. **79**:103, 1960) has been the principal means of obtaining nucleic acids from these organisms (Perry and Slade, Bacteriol Proc., p. 90, 1961; Pakula and Hulanicka, Bull. acad. polon. sci., Classe II **9(2)**:79, 1961). Since actinomycetin is not readily available, attempts were made to develop a simple procedure for lysing lactic acid bacteria.

Preliminary experiments revealed that *Streptococcus lactis*, *S. cremoris*, and *S. diacetilactis* were resistant to lysis by lysozyme, sodium lauryl sulfate, or deoxycholic acid when suspended in physiological saline or 0.1 M phosphate buffer (pH 7.0). However, when *S. diacetilactis* was grown in a medium containing 2% sodium citrate, nucleic acids could be recovered without the addition of any lytic agent. This effect of citrate was variable, because of the active citritase enzyme possessed by this organism (Sandine et al. J. Dairy Sci. **44**:1158, 1961).

This finding suggested that bacterial cells were sensitized to lysozyme by chelating agents. Such a phenomenon has been reported for gram-negative bacteria by Repaské (Biochim. et Biophys. Acta **30**:225, 1958). The lytic system developed by this investigator was therefore tested against several lactic acid bacteria.

Cells for lysis experiments were inoculated from lactic agar stabs (Elliker et al., J. Dairy Sci. **39**:1611, 1956) into 10-ml broth tubes of the same medium. After incubation for 12 hr at the optimal growth temperature, cells were centrifuged and washed twice. They were resuspended in 3.0 ml of the various lysing media; the complete system contained 100 μmoles of tris(hydroxymethyl)aminomethane (tris) buffer (pH 8.0), 100 μg of lysozyme, and 400 μg of ethylenediaminetetraacetic acid (EDTA). Lysis was measured by following the decrease in optical density from an initial value of about 0.7, using a Beckman model B spectrophotometer at 660 mμ.

Results of a typical experiment with *S. lactis* 27 are shown in Fig. 1. The accelerating effect of the EDTA, especially during the first 30 min of incubation, may be seen. Application of the system to other lactic acid bacteria revealed wide differences in sensitivity to lysis (Table 1). Although *S. lactis* and *S. diacetilactis* were resistant