SHORT COMMUNICATION

A Cellophane Membrane Method for Screening Auxotrophic Mutants of Photochromogenic Mycobacteria

By BOLDIZSÁR P. VAJDA

*Microbiological Research Group of the Hungarian Academy of Sciences,
Budapest 1529, Hungary*

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A high-resolution screening procedure for the selection of mutants was developed based on the ability of some strains of *Mycobacterium smegmatis* to produce pigment in the light. Out of 23000 colonies investigated, 17 auxotrophic mutants were isolated. Specific growth factor requirements for the mutants and the proportion of revertants were determined.

INTRODUCTION

The experimental genetics of mycobacteria is a somewhat neglected area of research. This can be attributed mainly to difficulties in handling mycobacteria. Conventional methods for the enrichment and selection of mutants are not applicable, e.g. the penicillin method of Davis (1948), or the replica-plating method of Lederberg & Lederberg (1952) which fails because of the extraordinarily dry character of mycobacterial colonies. As methods have been described for mutagenic treatment of mycobacteria to induce auxotrophic, antibiotic-resistant and pigment mutants (summarized by Koníčková-Radochová *et al.*, 1970) and for enrichment of mutants (Holland & Ratledge, 1971), my aim was to develop an efficient screening procedure for the selection of auxotrophic mutants.

Messer & Vielmetter (1965) devised a method for the visual identification of colonies of *Escherichia coli* and *Bacillus subtilis* auxotrophs. This was based on the fact that only growing cells were able to synthesize an inducible enzyme. Colonies were grown on membranes laid on the surface of agar medium so that they could be transferred, without damaging them, from one medium to another. In the final step, enzyme induction was carried out on the 'induction medium' and the colonies were stained for enzyme activity; auxotroph (non-growing) colonies were identified by being colourless.

This method was adopted but, instead of artificial staining, the ability of some strains of growing mycobacteria to produce an orange-red pigment when exposed to light was used to select the non-growing (colourless) mutant phenotype.

METHODS

Media. Minimal medium (MM) contained (g l⁻¹): NaCl, 1; (NH₄)₂SO₄, 3; glucose, 2; KH₂PO₄, 0.75; Tris, 3. The pH was adjusted to 7.2 with 11 M-HCl and 1 ml 1 M-MgCl₂ was added. Complete medium (YET) was MM supplemented with Difco yeast extract (3 g l⁻¹) and Difco Bacto-Tryptone (5 g l⁻¹). Agar plates were prepared by adding 1.2% (w/v) Difco Bacto-agar to MM or YET. Soft agar contained 0.7% (w/v) agar.

The bacterial strains were maintained on YET slants, solidified with 1.5% (w/v) agar.

The buffer used throughout was 0.05 M-Tris/HCl, pH 7.2, containing 4 mM-CaCl₂.

Preparation of the YET-cellophane agar plates. Cellophane membranes (9 cm diam.) were prepared from

commercial cellophane used for domestic purposes and sterilized by autoclaving. The wet membranes were laid on the surface of YET agar plates. It was important not to allow the membranes to get crumpled.

Organism and culture method. The prototrophic Rabinowitz strain of *Mycobacterium smegmatis* was used. Cells grown on a YET slant were inoculated into liquid YET medium supplemented with 0.1 % (v/v) Tween 80 (to minimize the clumping of cells) and grown at 37 °C on a shaking platform (60 oscillations min⁻¹).

Mutagenesis. Bacteria in the exponential phase of growth (A_{660} about 0.2; about 10^8 cells ml⁻¹) were diluted 100 and 1000 times, spread on YET-cellophane agar plates and irradiated with u.v. light (Uvilux, Labor MIM-Hungary; 253.7 nm, emitted power 12 W) at a 10^{-3} survival level.

Determination of the number of colony formers. Cells grown in liquid YET medium supplemented with 0.1 % (v/v) Tween 80 were collected by centrifuging and washed and resuspended in buffer. The cell suspension was serially diluted with buffer, and 0.1 ml samples of the dilutions were mixed with 2 ml melted (45 °C) MM or YET soft agar and poured on MM or YET agar. The plates were incubated at 37 °C. Under these conditions the colonies could be counted on the third day.

Tests for specific growth factors. Each isolated mutant strain was cultured in liquid YET medium supplemented with Tween 80 and then streaked on to MM agar plates supplemented with one of the following growth factors at the indicated concentration ($\mu\text{g ml}^{-1}$): adenine (50), alanine (50), arginine (50), biotin (1), cysteine (50), cytosine (50), folic acid (5), glutamic acid (50), guanine (50), histidine (50), inositol (5), isoleucine (50), leucine (50), lysine (50), methionine (50), sodium thiosulphate (50), nicotinic acid (0.5), pantothenic acid (10), riboflavin (2.5), tyrosine (50), thiamin (1), thymine (50), tryptophan (50), uracil (50). The test was considered positive if the mutant showed the same growth as the prototrophic control strain.

RESULTS AND DISCUSSION

The Rabinowitz strain of *M. smegmatis* used in this study for the isolation of auxotrophic mutants is photochromogenic. Colonies exposed to visible light produce an orange-red pigment, whereas in the dark the colonies remain white. I supposed that auxotrophic mutants unable to multiply in the absence of the required growth factor would not be able to produce the orange-red pigment. A complication of this argument is the possible production of pigment mutants which are white but prototrophic. These are not distinguishable but can be excluded at the first test for auxotrophy.

The mutagen-treated cells were grown in the dark on a cellophane membrane placed on complete agar until colonies appeared. The cellophane membrane carrying the small white mycobacterial colonies was then transferred to the surface of an MM agar plate and incubated for an additional day in the dark to exhaust the bacterial nutrient pools. The plate was then exposed to visible light for a few days (usually 3 d). After this prolonged incubation in the light, the white and usually (but not invariably) smaller colonies were picked out from the mass of orange-red colonies for further testing.

The number of colonies that can be distinguished on a single plate determines the resolution of the test and limits the efficiency of the procedure. Since in this method the colonies themselves are 'stained', the resolution of the test is very high. It was possible to isolate auxotrophs from plates carrying over 1000 colonies.

Of 23000 colonies screened, 624 were white. These were streaked on MM and YET agar plates. Of the 624 isolates, 101 were unable to grow on MM plates. This means that after u.v. treatment the proportion of the cells showing mutant character is about 4×10^{-3} .

At the beginning of this work, isolates were considered to be mutants if they produced 10^4 times more colonies on complete agar than on minimal agar after 3 d incubation. Out of the 101 isolates, 53 fulfilled this minimum requirement. However, if the incubation time was prolonged, slowly growing small colonies appeared on the MM plates, increasing in number until the seventh day. Thus, subsequently, only those isolates which showed no leakiness were considered as mutants. Of the 53 isolates, 17 proved to be non-leaky. The specific growth factor requirements and the proportion of revertants for these 17 mutants are shown in Table 1.

I tried to develop a 'direct' variant of the cellophane membrane mutant-screening method. In this, minimal agar supplemented with one growth factor was used instead of complete agar for the period of growth in the dark. Thus, of the auxotrophic mutants, only those

Table 1. *Growth factor requirement and the proportion of revertants of the isolated mutants*

The specific growth factor for each isolated mutant strain was determined by culturing the strains in minimal medium supplemented with one of the growth factors; the proportion of revertants was calculated by comparing the number of colonies in minimal and complete media.

Strain designation	Growth factor	Proportion of revertants
UA1	Arginine	$< 2.4 \times 10^{-9}$
UA12	Folic acid	4.6×10^{-7}
UA75	Histidine	$< 2.2 \times 10^{-7}$
UA80	Adenine or guanine	1.3×10^{-5}
UA104	Adenine or guanine	$< 9.1 \times 10^{-9}$
UA110	Histidine	3.5×10^{-6}
UA111	Adenine or guanine	5.1×10^{-8}
UA117	Histidine	1.8×10^{-6}
UA120	Folic acid	$< 3.9 \times 10^{-9}$
UA131	Histidine	$< 1.2 \times 10^{-8}$
UA140	Histidine	2.6×10^{-6}
UA143	Arginine	4.4×10^{-8}
UA158	Adenine or guanine	$< 1.0 \times 10^{-8}$
UA160	Leucine	4.6×10^{-8}
UA162	Methionine	$< 1.3 \times 10^{-9}$
UA163	Adenine or guanine	$< 2.1 \times 10^{-9}$
UA165	Histidine	$< 1.0 \times 10^{-7}$

requiring this growth factor would form colonies. By this 'direct' method, a methionine- and an arginine-requiring mutant strain were isolated.

Having obtained the mutant strains, I tested the reliability of the screening method. Wild-type and auxotroph cells were mixed in different ratios and the mixtures were spread on YET-cellophane agar plates. The procedure was carried out as described, but omitting the u.v.-irradiation, and the ratios of orange-red to white colonies were determined. There was good agreement between the calculated and experimental ratios of pigmented (wild-type) to non-pigmented (mutant) colonies. It appears, therefore, that the method described is an objective procedure for screening mutant colonies.

As the pigment-producing ability is not a unique feature of photochromogenic mycobacteria, the isolation of mutants in other pigment-producing bacteria would presumably be possible by the same method.

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REFERENCES

- DAVIS, B. D. (1948). Isolation of biochemically deficient mutants of bacteria by penicillin. *Journal of the American Chemical Society* **70**, 4267-4269.
- HOLLAND, K. T. & RATLEDGE, C. (1971). A procedure for selecting and isolating specific auxotrophic mutants of *Mycobacterium smegmatis*. *Journal of General Microbiology* **66**, 115-118.
- KONÍČKOVÁ-RADOCHOVÁ, M., KONÍČEK, J. & MÁLEK, I. (1970). The study of mutagenesis in *Mycobacterium phlei*. *Folia microbiologica* **15**, 88-102.
- LEDERBERG, J. & LEDERBERG, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *Journal of Bacteriology* **63**, 399-406.
- MESSER, W. & VIETMETTER, W. (1965). High resolution colony staining for the detection of bacterial growth requirement mutants using naphthol azo-dye techniques. *Biochemical and Biophysical Research Communications* **21**, 182-186.