A Procedure for Selecting and Isolating Specific Auxotrophic Mutants of *Mycobacterium smegmatis*

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SUMMARY

Mycobacterium smegmatis is mutated by exposure to nitrosoguanidine at 0.1 and 1.0 mg./ml. for 15 and 60 min. After incubation for 24 h. in a complete medium, to allow for phenotypic expression, isoniazid (at 0.1 mg./ml. for 24 h.) is used to reduce the numbers of unwanted mutants and surviving wild-type bacteria in a procedure analogous to penicillin selection with other bacteria.

INTRODUCTION

When mutants of *Mycobacterium phlei*, produced by a variety of treatments, were isolated without an enrichment procedure they required mainly glycine and serine for growth (Konichova-Radochova & Malek, 1969; Konichova-Radochova, Konicek & Malek, 1970). No technique, however, has previously been described for selecting and isolating specific auxotrophs of mycobacteria. Such a technique has now been evolved and is presented here. A preliminary account has already been published (Holland & Ratledge, 1970).

METHODS

Organism and growth. *Mycobacterium smegmatis* was grown on a minimal medium previously described (Ratledge & Hall, 1970), without removing trace metals. The medium, when required, was solidified with high purity agar (Oxoid no. 1, Oxoid Laboratories) at 1.5 % (w/v). Nutrient broth and nutrient agar (Difco Laboratories) were also used. Supplements were added to sterile media from sterile stock solutions. Tween 80 was autoclaved as a 10 % (v/v) solution in water.

Bacteria in liquid media were always continuously shaken at 250 rev./min. All cultures were incubated at 37°. For determining viable bacterial count, serial dilutions were made in 0.85 % (w/v) NaCl + 1 % (v/v) Tween 80 and samples (0.2 ml.) dispersed on solid medium with a glass spreader. The turbidity of cultures at 660 nm. was used whenever a quick indication of the viable count was necessary.

Mutagenesis and phenotypic expression of mutants. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) was used according to Adelberg, Mandel & Chen (1965). For optimum results, bacteria were harvested by centrifuging near the end of log-phase growth, washed with water and resuspended in minimal medium at pH 6.3 + 1 % (v/v) Tween 80 to a concentration of about 10⁸ bacteria/ml., with nitrosoguanidine usually at 100 or 1000 µg./ml. being added beforehand. After incubation, samples (10 ml.) were membrane-filtered and the bacteria washed with 0.2M KH₂PO₄ + KOH buffer, pH 6.3. The membrane was transferred into 100 ml. nutrient broth + 1 % (v/v) Tween 80 in a 250 ml. conical flask along with 10 to

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20 glass balls (0.5 cm. diam.) to dislodge bacteria from the filter and then shaken at 37° for 24 h.

Selection of mutants. Penicillin cannot be used for enrichment of mutants of mycobacteria as they are insensitive to it (Konicek & Malek, 1968). Isonicotinic acid hydrazide (isoniazid) is, however, highly active against mycobacteria killing only actively growing bacteria (Winder, 1964). A 10 ml. sample of bacteria, following nitrosoguanidine treatment and a period for phenotypic expression, was membrane-filtered, washed well with buffer and transferred into 100 ml. of minimal medium with 1 % (v/v) Tween 80, 100 µg. isoniazid/ml. and every chemically defined growth factor listed below except the one for which auxotrophic mutants were required. Glass balls were again added to dislodge bacteria from the membrane. The medium was incubated for 24 h. Bacteria (usually a 10 ml. sample) were collected and washed as before, then resuspended in the usual manner in 100 ml. of minimal medium with 1 % (v/v) Tween 80 and the growth factor for the required auxotroph. The medium was shaken for 24 h. Samples (1 ml.) were serially diluted (see above), plated out on solidified minimal medium containing 1 % (v/v) Tween 80 and the growth factor for the required auxotroph. After incubation for 3 days mucoid colonies developed and replica plating of those plates with 50 to 150 colonies was carried out following the procedure of Lederberg & Lederberg (1952).

Growth factors. The following growth factors (final concentrations in µg./ml.) were included in enrichment medium containing isoniazid: L-alanine (10), L-arginine (10), DL-aspartic acid (10), L-cystine (10), L-glutamate (10), glycine (10), L-histidine (10), L-hydroxyproline (10), L-isoleucine (10), L-leucine (10), L-lysine (10), L-methionine (10), L-phenylalanine (10), L-proline (10), L-serine (10), L-threonine (10), L-tryptophan (10), L-tyrosine (10), L-valine (10), shikimic acid (15), salicylic acid (10), riboflavin (1), thiamine.HCl (1), biotin (1), nicotinamide (2), 4-aminobenzoate (2), pantothenic acid (1), pyridoxamine.HCl (0.8), folic acid (0.2), inositol (2), adenine (20), guanine (20), uracil (20), xanthine (20), thymidine (20), cytosine (20).

RESULTS AND DISCUSSION

Successful auxotroph selection depends upon bacteria being maintained in a dispersed form. Clumps of *Mycobacterium smegmatis* were disaggregated when 1 % (v/v) but not 0.05 % Tween 80 was incorporated in media, but constant shaking was also needed to maintain dispersion. Under these conditions sustained logarithmic growth, with a mean generation time of 3 h., occurred in both minimal medium and nutrient broth.

Optimum concentration of mutagen and most suitable medium for exposure to mutagen. The decrease in viability of *Mycobacterium smegmatis* when treated with nitrosoguanidine at either 100 or 1000 µg./ml. in minimal medium or at 60 µg./ml. in nutrient broth was followed (see Table 1). The lethal effect of nitrosoguanidine on bacteria suspended in broth was, however, considered too great and all subsequent experiments were carried out using minimal medium with nitrosoguanidine at two concentrations (100 and 1000 µg./ml.).

Determination of optimum time for exposure to mutagen. Using the above conditions the maximum number of auxotrophic mutants were produced after 45 min. contact with nitrosoguanidine at both 100 and 1000 µg./ml. (Table 1). However, we recommend that bacteria be exposed for two periods – 15 min. and about 60 min. – for each of the two concentrations of mutagen.

Determination of the optimum time for phenotypic expression. The optimum time to leave the mutated bacteria in complete medium for phenotypic expression was 24 h. This applied to all bacteria no matter how long they had been in contact with nitrosoguanidine.

Without this step, the number of mutants isolated was about 50 % less; if bacteria were incubated for longer than 24 h. the number of isolates again decreased.

Non-selective isolation of mutants. Without isoniazid to select specific auxotrophs, mutants could be isolated by plating out samples from the medium used for phenotypic expression on to nutrient agar + 1 % (v/v) Tween 80. Colonies were subsequently replica-plated on minimal medium containing either all amino acids, purines + pyrimidines or vitamins. Combinations

Table 1. *Survival of Mycobacterium smegmatis and production of mutants after exposure to nitrosoguanidine*

Bacteria were suspended in either nutrient broth + 1 % (v/v) Tween 80 medium or in a minimal medium + 1 % (v/v) Tween 80 to a density of 10^8 bacteria/ml. Nitrosoguanidine was then added. Cultures were shaken at 250 rev./min. at 37°. For total viable count of surviving bacteria, samples (1 ml.) were immediately diluted in Tween 80 + saline (see Methods) and plated out on nutrient agar. For count of mutants, samples (10 ml.) were processed as given in Methods and, after incubation in nutrient broth for 24 h., mutants were isolated without the isoniazid selection procedure.

Time of treatment (min.)	Nitrosoguanidine at				
	60 μ g./ml. (in nutrient broth) Survivors (%)	100 μ g./ml. (in minimal medium)		1000 μ g./ml. (in minimal medium)	
		Survivors (%)	Mutants in surviving population (%)	Survivors (%)	Mutants in surviving population (%)
10	20	—	—	—	—
15	—	26	1.3	16	2.3
20	1	—	—	—	—
30	0	14	1.7	7	4.3
45	—	12	2.8	3	10
60	—	9	2.4	1	8.2

Table 2. *Effect of isoniazid on the recovery of the wild-type and an amino acid auxotroph from a mixed culture in minimal medium*

Both wild-type and mutant were grown separately on nutrient broth + 1 % (v/v) Tween 80, membrane filtered, washed and then mixed in minimal medium containing 1 % (v/v) Tween 80 and isoniazid. The mixed culture was shaken at 37° for 30 h.

	Viable count (bacteria/ml.) of	
	Auxotroph*	Wild-type
Initially in mixed culture...	3.5×10^8	2.35×10^6
After exposure to isoniazid at:		
0 μ g./ml.	7.9×10^5	2.3×10^8
10 μ g./ml.	5.4×10^6	3.8×10^6
100 μ g./ml.	3.0×10^6	1.0×10^4
1000 μ g./ml.	3.5×10^6	3.9×10^3

* The exact nutritional requirement was not defined beyond the need for an amino acid.

of these growth factors were also used. Although the exact nutritional requirement of each mutant was not defined further, only a few mutants showed a requirement for more than one group of growth factors. In a typical experiment 55 % of the mutants required an amino acid, 19 % a purine or pyrimidine and 16 % a vitamin. Auxotrophic mutants requiring asparagine were obtained by substituting $(\text{NH}_4)_2\text{SO}_4$ at 0.5 g./l. for L-asparagine in the minimal medium and mutants resistant to streptomycin, isoniazid and *p*-aminosalicylate

could also be obtained. Mutants with an altered chromogenesis and also a few temperature-sensitive mutants were noted.

Isolation of specific auxotrophs. Isoniazid at various concentrations, up to 1000 µg./ml., did not produce any significant decrease in the viable count of an auxotroph, obtained by the non-selective method, whereas at 100 or 1000 µg./ml. it produced a substantial killing of the wild-type (Table 2). The resistance of the auxotroph to isoniazid can be attributed to the inability of the drug to be bactericidal to non-growing bacteria (Winder, 1964). Thus isoniazid can be used to enrich a mixed culture in favour of any auxotroph which may be present and its use with mycobacteria parallels that of penicillin with other bacteria.

The efficacy of this method in isolating specific mutants has been demonstrated by the facility with which auxotrophs of *Mycobacterium smegmatis* requiring salicylic acid, tyrosine, tryptophan and phenylalanine, as well as some demonstrating a requirement for shikimic acid, have been obtained. The number of colonies showing inability to grow on minimal medium but being able to grow on the same medium plus the specific growth factor was usually between 0.2 % and 0.9 % of the final colony population.

Isolated auxotrophs have shown a high degree of stability without any change in nutritional requirements over a period of 12 months. There is no reason why this technique could not be applied with equal success to other species of mycobacteria.

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