Use of Trimethoprim to Obtain Thymine-requiring Mutants of *Streptococcus faecalis*

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(Received 24 April 1972; revised 24 August 1972)

INTRODUCTION

The use of a folic acid antagonist to isolate thymine-requiring (*thy*) mutants was first employed by Okada, Yanagisawa & Ryan (1960) with *Escherichia coli*. *Thy* mutants arise when wild-type bacteria are grown in medium containing the drug (aminopterin or trimethoprim) and thymine (or thymidine). The mutants lack thymidylate synthetase (Barner & Cohen, 1959) and are able to incorporate exogenous thymine (Crawford, 1958) which gives them a selective advantage over the wild-type bacteria (Bertino & Stacey, 1966; Wilson, Farmer & Rothman, 1966). This is now an established technique which has also been used successfully with *Salmonella typhimurium* (Okada, Homma & Sonohara, 1962), *Bacillus megaterium* (Wachsman, Kemp & Hogg, 1964), *Aerobacter aerogenes* (Harrison, 1965) and *B. subtilis* (Farmer & Rothman, 1965).

This report describes the isolation of *thy* mutants of *Streptococcus faecalis* by a modification of the trimethoprim technique of Stacey & Simson (1965).

METHODS

Strains and growth conditions. *Streptococcus faecalis* NCTC 775 (from the National Collection of Type Cultures) and *S. faecalis* EB/F/30/82 (from P. H. A. Sneath) were grown in a chemically defined medium (SF35) which was modified from media described by VanDe-mark (1950), McCoy & Wender (1953), Stonehill & Hutchison (1966) and Clark, Witter & Ordal (1968).

Salts A (made up as a 20-fold concentrated stock solution containing per l distilled water): K_2HPO_4 , 140 g; KH_2PO_4 , 50 g; tri-sodium citrate, 50 g; sodium acetate, 10 g; $(NH_4)_2SO_4$, 20 g; L-arginine, 2 g; L-cysteine, 4 g; L-glutamic acid, 8 g; L-leucine, 2 g; L-*iso*-leucine, 4 g; L-lysine, 4 g; L-serine, 2 g; L-threonine, 2 g; DL-valine, 2 g; glycine, 1.2 g; L-histidine, 1.2 g; L-methionine, 1.2 g; L-phenylalanine, 800 mg; L-tryptophan, 800 mg; L-tyrosine, 400 mg; $MgSO_4 \cdot 7H_2O$, 250 mg.

Salts B (made up as a 100-fold concentrated stock solution containing per l 0.067M-potassium phosphate buffer): thiamine HCl, 40 mg; calcium pantothenate, 40 mg; nicotinic acid, 40 mg; pyridoxal HCl, 40 mg; riboflavin-5-phosphoric acid, 100 mg; adenine sulphate, 1 g; guanine HCl, 2 g; biotin, 1 mg; folic acid, 200 μ g. Guanine HCl and riboflavin-5-phosphoric acid caused precipitation when included in Salts B so they were made up separately, the guanine HCl being dissolved in hot acidified distilled water to make a 100-fold concentrate and the riboflavin in phosphate buffer as a 500-fold concentrate.

Salts C (made up as a 100-fold concentrate stock solution containing per l distilled water): $MnSO_4 \cdot 4H_2O$, 2 g; NaCl, 2 g; $FeSO_4 \cdot 7H_2O$, 2 g; tri-sodium citrate, 8 g. When required thymine was made up as a 20-fold concentrate by dissolving it in hot distilled water and the

trimethoprim (2,4-diamino-5-(3',4',5'-trimethoxybenzyl) pyrimidine) as a 100-fold concentrate in acidified distilled water.

All the solutions were sterilized by filtration through 0.8 and 0.2 μm Gelman membrane filters (Hawkesley and Sons Ltd., Lancing, Sussex) with the exception of Salts A which was autoclaved at 10 lb/in² for 10 min. The stock solution of riboflavin was stored at room temperature (20 °C) to avoid precipitation, Salt solution A was stored at -20 °C to retard precipitation of sulphur-containing compounds, and the other solutions were stored in the dark at 0 to 4 °C. The complete medium at pH 7 was made up by adding the stock solutions to a glucose solution (final concentration 2 %). Solid medium included 1.5 % (w/v) Ionagar (Oxoid).

Single colonies from stock SF35 agar slopes were inoculated into 50 ml SF35 broth and incubated for 15 to 18 h at 37 °C. The bacteria were then harvested by centrifugation, washed twice in phosphate buffer and finally resuspended in 10 ml of the same buffer. The resulting bacterial suspension was used in the experiments as a 1 % (v/v) inoculum.

Growth measurements. Viable counts were made by diluting the bacteria in 0.067 M-phosphate buffer and either inoculating 0.1 ml of an appropriate dilution on to 10 ml SF35 agar plates which had been overdried for 2 h at 37 °C or by making pour plates using 1 ml of a dilution in 10 ml SF35 agar at 50 °C. Colonies were counted after 48 h incubation at 37 °C.

Extinction measurements were made at 553 nm with a Vitatron colorimeter (Fisons Scientific Apparatus Ltd, Loughborough, Leicestershire) using the riboflavin stock solution diluted to its working strength in distilled water as the blank.

RESULTS

When these strains of *Streptococcus faecalis* were incubated with 10 μg trimethoprim/ml and 50 μg thymine/ml growth occurred almost immediately, without the customary 2- to 3-day lag, maximum turbidity was reached after 10 h and no mutants were detectable among approximately 5000 colonies screened for thymine dependence by replica plating. In the absence of thymine the bacteria were sensitive to trimethoprim losing viability after 2 h (Fig. 1). Wild-type bacteria were therefore able to utilize exogenous thymine to reverse trimethoprim inhibition and selection of *thy* mutants was prevented.

The presence of adenine and guanine in the medium decreases the inhibitory effect of trimethoprim (Miovic & Pizer, 1971) and, since these *Streptococcus faecalis* strains could grow without them, they were removed from the medium and the selection procedure repeated. Thymine was then unable to reverse trimethoprim inhibition but there was no increase in viable count even after 7 days. Thus the purines were obviously required in trimethoprim-containing medium before bacteria of any genotype would grow and yet added thymine allowed *Thy*⁺ bacteria to overcome trimethoprim inhibition and grow almost normally preventing the selection of *thy* mutants.

To examine whether wild-type bacteria could still overcome trimethoprim inhibition with thymine after prior treatment with the drug alone, *Thy*⁺ strains were incubated in complete medium with 10 μg trimethoprim/ml for 9 h until the viable count and extinction remained constant (arrow, Fig. 1). At this point a portion of the culture was inoculated into fresh medium containing both the drug and 50 μg thymine/ml. After a further two days of incubation a fully turbid culture was obtained and replica plating of colonies from surviving bacteria showed that 31 % were *thy* mutants. They had a low thymine requirement (2 μg /ml) and produced easily recognizable colonies on SF35 agar replica plates which were smaller

Fig. 1. Growth of *Streptococcus faecalis* EB/F/30/82 at 37 °C in SF35 broth (—○—○—) or SF35 broth containing 50 µg thymine/ml (—●—●—); 10 µg trimethoprim/ml (—□—□—); 10 µg trimethoprim/ml and 50 µg thymine/ml (—■—■—). Viable counts were made by the pour plate technique.

than normal and semitransparent. These *thy* bacteria were resistant to trimethoprim which is characteristic of mutants selected in this way (Stacey & Simson, 1965). Mutants arose whether or not trimethoprim was present during the second period of incubation which may indicate its persistence in the bacteria; however no growth occurred if thymine was absent.

It was tempting to infer that the viable bacteria surviving trimethoprim treatment (about 0.5 % of the original population) contained *thy* mutants resistant to the drug. However, as found by Okada *et al.* (1960, 1961), no such mutants could be isolated from approximately 5000 colonies derived from this fraction. These bacteria therefore appear to need an initial treatment with trimethoprim before *thy* mutants have a selective advantage over wild-type cells during subsequent growth with thymine.

DISCUSSION

These wild-type strains of *Streptococcus faecalis* are unusual in that they were able to utilize exogenous thymine and thus overcome trimethoprim inhibition. This may be due to active deoxyribonucleotide catabolism in these strains which is usually absent in wild-type

bacteria and prevents utilization of exogenous thymine (Beacham, Barth & Pritchard, 1968; Beacham & Prichard, 1971). An alternative explanation may lie in the use of a complex growth medium for these streptococci which have fastidious nutritional requirements. Koch & Burchall (1971) reported that *Escherichia coli* B could overcome trimethoprim inhibition with exogenous thymidine when grown in a complex medium containing amino acids, ribosides and vitamins.

A second unusual feature of these streptococci is that they gave rise to mutants with a low thymine requirement after trimethoprim selection. Usually mutants selected in this way have a high requirement for thymine ($> 20 \mu\text{g/ml}$) (Stacey & Simson, 1965; Okada, 1966). The low requirement is considered to be the result of a second mutation at a locus unrelated to *thy* (Alikhanian, *et al.* 1966; Eisenstark, Eisenstark & Cunningham, 1968) and a second selection procedure is needed (Harrison, 1965) to obtain these double mutants which have no phosphodeoxyribomutase or deoxyriboaldolase activity (Munch-Petersen, 1968). This lessens competition for deoxyribose-1-phosphate in the cell and lower concentrations of exogenous thymine suffice (Beacham *et al.* 1968; O'Donovan & Neuhard, 1970). However the large numbers of *thy* mutants of *Streptococcus faecalis* arising after trimethoprim treatment does not accord with the selection of double mutants.

I am grateful to the Wellcome Foundation for their gift of trimethoprim and to Dr I. ab I. Davies for his very helpful discussion.

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