The Induction of Mutants of *Acinetobacter calcoaceticus* NCIB8250 and their Selection by Vancomycin

By E. F. AHLQUIST*, C. A. FEWSON AND D. A. RITCHIE

Departments of Biochemistry and Virology, University of Glasgow, Glasgow G12 8QQ

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SUMMARY

The mutagenic and lethal effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), ethyl methanesulphonate (EMS), ultraviolet light irradiation and near-ultraviolet light irradiation with 8-methoxypsoralen on the bacterium *Acinetobacter calcoaceticus* NCIB8250 were examined. The production of auxotrophic mutants was used as a measure of mutagenic efficiency. Under appropriate conditions all four agents were mutagenic. EMS and NTG although more effective than irradiation, did not cause such a high frequency of mutation as has been observed with other bacteria. A combination of vancomycin and penicillin V gave enrichment of non-metabolizing bacteria and optimum conditions were found for the use of these compounds in a selection technique.

INTRODUCTION

Increasing interest in the biochemistry and physiology of *Acinetobacter calcoaceticus* has led to the search for mutants affected in various metabolic functions. Several workers (e.g. Cánovas & Stanier, 1967; Livingstone *et al.* 1972; Sawula, Crawford & Irving, 1972; Herman & Juni, 1974) have obtained various classes of mutants of *A. calcoaceticus*, usually after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) using conditions similar to those described by Adelberg, Mandel & Chen (1965) for *Escherichia coli*. However, methods designed for the induction and selection of mutants of one micro-organism are seldom the most appropriate ones for use with another micro-organism (Hopwood, 1970) and in preliminary studies with *A. calcoaceticus* NCIB8250 we found that conventional techniques for bacterial mutagenesis produced rather low yields of mutants. Consequently, we examined the lethal and mutagenic effects of various agents on *A. calcoaceticus* NCIB8250 and developed an enrichment system suitable for use with this and other strains of *A. calcoaceticus*. This work forms part of a study undertaken to provide a collection of mutants for the analysis of the metabolism of aromatic compounds.

METHODS

Organisms. Strain 73 was kindly supplied by Dr J. L. Cánovas. Strain u4K, auxotrophic for tyrosine, was derived from wild-type NCIB8250 by u.v. irradiation in a preliminary experiment. Other strains were obtained from the National Collection of Industrial Bacteria (NCIB), Torry Research Station, Aberdeen, or from the American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A.

* Present address: Biological Laboratories, University of Kent, Canterbury CT2 7NJ.

Procedures for the growth and maintenance of bacteria and the compositions of basal and salts media have been described (Kennedy & Fewson, 1968; Livingstone *et al.* 1972).

Measurement of bacterial numbers. Viable bacterial titres in liquid cultures were determined by spreading samples diluted in basal medium on to nutrient agar plates. Colonies were counted after incubation for 1 to 2 days at 30 °C.

The proportion of auxotrophs was measured by replicating from nutrient agar plates on to 10 mM-succinate-salts agar on which auxotrophs are unable to grow (Lederberg & Lederberg, 1952). Non-replicating colonies were checked by streaking on 10 mM-succinate-salts agar. Usually the minimum number of auxotrophs which could be determined was about 0.05 % of the total viable bacteria; proportions less than this were recorded as zero.

Auxotrophic requirements were determined by the pool-plate method of Holliday (1956). Relative enrichment was calculated as

$$\frac{\% \text{ mutant survivors of antibiotic treatment}}{\% \text{ wild-type survivors of antibiotic treatment}}$$

Treatment with mutagenic agents. Sterile techniques were used throughout. Before treatment, bacteria were grown in 5 mM L-glutamate-salts medium, harvested in exponential phase, concentrated by centrifugation, and resuspended in basal medium at 5×10^9 bacteria/ml. After treatment with the mutagens, bacteria were diluted and plated on nutrient agar to permit replication and enumeration of auxotrophs.

(i) Ethyl methanesulphonate (EMS; 625 mM, filtered through 0.22 μ m Millipore filters) was added to bacterial suspensions at various concentrations. Following incubation at 30 °C without shaking, the cells were centrifuged and washed once in basal medium.

(ii) NTG was added to bacteria suspended in 10 mM-sodium citrate or -tris-maleate buffer, pH 6, to give a total volume of 10 ml. Cultures were incubated at 30 °C in 50 ml Erlenmeyer flasks and at various times samples were diluted in basal medium.

(iii) Ultraviolet light irradiation with a Hanovia model 13 bactericidal lamp (Engelhard Hanovia Lamps, Slough, Buckinghamshire) was carried out with 10 ml suspension magnetically stirred in glass Petri plates. All manipulations were done at room temperature with illumination from a sodium lamp to prevent photoreactivation.

(iv) 8-Methoxypsoralen (1 ml of a solution containing either 1.0 or 0.1 mg/ml dissolved in ethanol) was added to 9 ml suspensions which were incubated for 45 min at room temperature, and then irradiated in stirred glass Petri dishes with a 125 W GEC black lamp at a distance of 25 cm. A sodium lamp was used for all manipulations to prevent extra irradiation from fluorescent lamps.

Materials. NTG was bought from Koch-Light, EMS, neomycin and 8-methoxypsoralen from Sigma, vancomycin and D-cycloserine from Eli Lilly & Co. Ltd, Basingstoke, Hampshire, and cooked meat medium (CM82), nutrient broth (CM1) and nutrient agar (CM3) from Oxoid. Penicillin V was a gift to Dr W. H. Holms of the Biochemistry Department from ICI (Pharmaceuticals Division) Ltd, Alderley Park, Cheshire, sodium ampicillin was kindly supplied by Dr A. Livingstone, Beecham Pharmaceuticals, Worthing, Sussex, and carbenicillin by the Department of Bacteriology and Immunology, University of Glasgow. Other reagents were purchased from BDH, or were as described by Kennedy & Fewson (1968).

Fig. 1. Inactivation and mutagenesis of *A. calcoaceticus* NCIB8250. Suspensions containing approximately 5×10^8 bacteria/ml were incubated with each of the mutagenic agents and samples taken at intervals for viable counts and determination of proportion of auxotrophic mutants. The values given on the graphs represent the number of auxotrophs as a percentage of the surviving bacteria; n.d. = not determined; absence of a value indicates that no auxotrophs were found (i.e. usually $< 0.05\%$). (a) NTG at 10 (○), 100 (●) or 1000 (■) µg/ml. (b) EMS at 20 (○), 45 (●), 75 (■) or 95 (△) mM. (c) Ultraviolet irradiation at distances of 80.8 (○), 40.4 (●) or 20.2 (■) cm from light source. (d) Near-u.v. irradiation in the presence of 8-methoxypsoralen at 0.01 (○; auxotrophs not determined) or 0.1 (●) µg/ml.

RESULTS AND DISCUSSION

Induction of mutants

The method for measuring mutagenicity was based on the forward mutation rate from prototrophy to auxotrophy, i.e. the ability to grow on nutrient agar but not on succinate-salts medium. This was considered to give a better indication of general mutagenic activity than the more convenient method of measuring the reversion rate of a particular mutant which may give an unrepresentative assessment of mutation.

In a series of experiments (see Fig. 1), EMS produced the greatest proportion of auxotrophs of all the mutagenic agents tested. In routine use, mutagenesis with 40 to 75 µg EMS/ml for 18 or 25 h led to the appearance of between 0.7 and 16% of auxotrophs. Preliminary experiments showed no decrease in bacterial viability after 90 min incubation with concentrations of EMS below 50 mM, but inactivation proceeded rapidly at higher concentrations. The short treatments suitable for mutagenesis of *E. coli* and *Salmonella typhimurium* (Loveless & Howarth, 1959) were not effective with *A. calcoaceticus* which required more drastic treatments as used for *Corynebacterium* vÚA9366 by Nečásek, Pikálek & Drobník (1966).

NTG was almost as effective as EMS (Fig. 1); in other experiments, treatment with from 250 to 1000 µg/ml for 30 to 120 min consistently produced between 0.3 and 4% of auxotrophs in the surviving bacteria. Again the proportion of auxotrophs induced under given

Table 1. *Effects of antibiotics on survival of A. calcoaceticus* NCIB8250 in various media

Inocula of wild-type and strain-U4K (tyrosine-requiring) bacteria were grown separately in either nutrient broth or salts medium with an appropriate carbon source (5 mM), and tyrosine (100 µg/ml) in the case of U4K. Bacteria were harvested and inoculated into separate test media (5 mM-benzyl alcohol or phenylacetate or 10 mM-succinate or DL-malate) without tyrosine, to give a concentration of about 7×10^7 bacteria/ml. After shaking at 30 °C for 1 h, antibiotics (vancomycin at 500 µg/ml and penicillin V at 1000 µg/ml) were added and incubation continued for a further 17 h when samples were assayed for viable bacteria.

Antibiotic	Medium for growth of inoculum	Carbon source in test medium	Survivors (%)		Relative enrichment
			Wild type	U4K	
Vancomycin	L-Glutamate-salts	Benzyl alcohol	0.004	0.8	200
Penicillin V	L-Glutamate-salts	Benzyl alcohol	0.6	100	167
Vancomycin + penicillin V	L-Glutamate-salts	Benzyl alcohol	0.002	18.7	9350
	Nutrient broth	Benzyl alcohol	n.d.	6.9	—
	Benzyl alcohol-salts	Benzyl alcohol	0.009	0.0003	0.15
	Nutrient broth	Phenylacetate	n.d.	6.2	—
	Phenylacetate-salts	Phenylacetate	0.001	0.009	9
	Nutrient broth	Succinate	0.002	0.004	2
	Nutrient broth	DL-Malate	0.001	0.009	9

n.d., Not determined.

conditions was lower than that recorded for several other organisms (e.g. Adelberg *et al.* 1965), although Deliç, Hopwood & Friend (1970) found that quite severe treatment was necessary for *Streptomyces coelicolor*. Citrate and tris-maleate buffers as well as basal medium gave very similar results at pH 6, and the inclusion of glutamate as a growth substrate during mutagenesis had little effect.

Neither u.v. irradiation nor near-u.v. irradiation in the presence of 8-methoxypsoralen (Townsend, Wright & Hopwood, 1971) produced many auxotrophs. Neither near-u.v. irradiation nor 8-methoxypsoralen was mutagenic when used separately.

Enrichment of mutants

Preliminary experiments showed that several compounds, e.g. penicillin G, D-cycloserine and neomycin which have been used for the selective enrichment of non-growing organisms of other species (e.g. Schlegel & Jannasch, 1967) gave little or no enrichment with *A. calcoaceticus* NCIB8250. In addition, treatment of strain NCIB8250 with ampicillin plus D-cycloserine by the method described by Herman & Juni (1974) for their strain 78 gave no enrichment of non-growing bacteria since no more than 10 % of the bacteria were killed when growing in either 10 mM-succinate or 10 mM DL-malate-salts medium. However, vancomycin (optimally at 500 µg/ml), penicillin V (optimally at 1000 µg/ml) (Table 1; lines 1 and 2) and carbenicillin (1000 µg/ml) all gave some enrichment of the tyrosine auxotroph U4K compared with the wild type when grown for 17 h in 5 mM benzyl alcohol-salts medium. Tests with combinations of these antibiotics revealed that vancomycin with penicillin V could produce a relative enrichment of the auxotrophic mutant of over 9000 (Table 1; line 3). Reconstitution experiments subjecting mixtures of wild type and U4K to vancomycin plus penicillin V resulted in the degree of enrichment expected from separate treatment of the two strains. Penicillin V appeared to exert a protective effect on the mutant (Table 1; lines 1 and 3), possibly by interacting with vancomycin (Perkins, 1969).

The results in Table 1 show that metabolism, but not necessarily growth, is required for inactivation. Thus U4K was killed to about the same extent as the wild type in succinate- or DL-malate-salts media; both of these carbon sources are metabolized by constitutive enzymes immediately after growth in nutrient broth. In the case of benzyl alcohol and phenylactate, however, which require inducible enzymes for their metabolism, U4K was inactivated only if previously induced by growth on these compounds.

The wild type in basal medium without a carbon source was killed to the same low extent as was U4K in medium which contained non-metabolizable carbon sources and no tyrosine.

Both vancomycin and penicillin V affect cell wall synthesis, but their precise mechanism of action in *A. calcoaceticus* is unknown and effective concentrations are much higher than normally used with sensitive Gram-positive bacteria (Gale *et al.* 1972).

The optimum concentration of carbon source in the enrichment medium was equivalent to about 30 to 40 mg-atom carbon/l (e.g. 5 mM-benzyl alcohol, 10 mM-succinate). A time course of antibiotic action showed that treatment for 17 h gave maximum enrichment. A period of preincubation in the enrichment medium for 1 h before addition of antibiotics was necessary to minimize the killing of mutants; preincubation for only 30 min led to considerably more killing, possibly due to metabolism of endogenous materials, but preincubation for times up to 7 h gave no advantage over 1 h. An important qualification for success of the enrichment procedure after mutagenesis was to ensure suitable concentrations of live and dead bacteria; the number of mutagen-killed bacteria must be less than 10^9 /ml and the number of live bacteria at least equal to the number of dead bacteria. The deleterious effect of large numbers of dead bacteria is probably due to leakage of materials leading to cross-feeding.

Applicability of the method

Using these results, we designed the following method for isolating mutants of *A. calcoaceticus* NCIB8250. Bacteria were grown in 5 mM L-glutamate-salts medium, harvested in exponential phase, concentrated by centrifugation, and resuspended in basal medium to 5×10^9 bacteria/ml. The appropriate mutagenic treatment was carried out and the washed bacteria resuspended in basal medium. This suspension was used to inoculate 40 ml of a suitable medium (e.g. 5 mM L-glutamate-salts medium) and the bacteria were grown for 17 h at 30 °C with shaking to allow phenotypic expression. In some cases addition of carbon sources (e.g. benzoate) was then made and incubation was continued for 2.5 h to allow induction of the corresponding enzymes in the wild-type strain. The bacteria were then harvested by centrifugation, washed and suspended in 10 ml of basal medium. Portions (1 ml) of the suspension were used to inoculate 40 ml volumes of basal medium containing the carbon source appropriate to the particular enrichment. The cultures were then starved by shaking at 30 °C for 1 h. Vancomycin and penicillin V were added and the cultures shaken at 30 °C for 17 h. After this the bacteria were harvested, washed in chilled nutrient broth, and resuspended in nutrient broth for identification of mutants.

The mutagenic techniques described in this paper, followed by enrichment with vancomycin plus penicillin V, have been used to isolate mutants of NCIB8250 auxotrophic for amino acids (e.g. cysteine, isoleucine, isoleucine-valine or tyrosine) or blocked at various stages in the catabolism of benzyl alcohol (E. F. Ahlquist, V. Rowell, C. A. Fewson & D. A. Ritchie, unpublished observations). In these cases it was not possible to measure the relative enrichment with any certainty because the mutant frequency before enrichment was extremely low. However, there clearly was enrichment; for example, in one experiment no mutants were detected among approximately 8000 colonies tested before enrichment

whereas after treatment with vancomycin plus penicillin V, between 0.5 and 1.3 % of the surviving organisms had lesions in the oxidation of benzyl alcohol (benzyl alcohol or benzaldehyde dehydrogenase or enzymes of the catechol pathway).

In a few experiments mutants were selected by growth for 3 h in the presence of vancomycin (500 µg/ml) without penicillin V. In one case, wild-type bacteria were subjected to u.v. irradiation to give 2 % viable survivors; this treatment gives auxotrophic mutants at too low a frequency to be detected (see Fig. 1). After one cycle of vancomycin selection the frequency of auxotrophs was 0.35 % (10 colonies of 2850 tested) and following a second 3 h vancomycin selection this frequency had risen to 7.4 % (146 colonies of 1950 tested).

The vancomycin-penicillin technique was tested with other strains of *A. calcoaceticus* by comparing the bactericidal effect in salts medium without carbon source with that in 10 mM-succinate-salts medium. The ratio of survivors in the two media, i.e. the apparent enrichment, was calculated for each strain and found to be: NCIB8250, 4875; NCIB9115, 400; strain 73, 241; NCIB9019, 125; NCIB10553, 82; NCIB9017, 24; ATCC23055, 20; NCIB9299, 1; NCIB9205, 0.6. This method is therefore suitable for use with some other strains of *A. calcoaceticus*, and might even be improved by minor modifications appropriate to the strain. However, the wide spectrum of properties of this species indicates that no method is likely to be successful with all strains. This is exemplified by the contrasting degrees of enrichment achieved with strains NCIB8250 and NCIB10553, shown by Grant (1973) to be closely related, and by a comparison of the effects of ampicillin plus D-cycloserine on strains 78 (Herman & Juni, 1974) and NCIB8250.

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