SHORT COMMUNICATION

Induced Mutagenesis in *Rhizobium trifolii*

By DEIRDRE A. WALTON*† AND B. E. B. MOSELEY

Department of Microbiology, University of Edinburgh, College of Agriculture,
West Mains Road, Edinburgh EH9 3JG

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The efficiency of a variety of common mutagens in producing mutation in *Rhizobium trifolii* P3 was examined. Ethyl methanesulphonate, methyl methanesulphonate, decarbamoyl mitomycin C, nitrous acid and gamma radiation did not mutate *R. trifolii* P3. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and ultraviolet radiation were both mutagenic, the former being the more effective. Transposon mutagenesis with Tn5 yielded the same frequency and range of auxotrophs as did MNNG.

INTRODUCTION

The successful isolation of mutants having a non-selectable phenotype, e.g. ineffective or non-infective mutants of *Rhizobium*, depends upon a high induced mutation frequency. It is often assumed that mutagens effective for one or more species of bacteria will be so for other species.

Although the efficacies of a variety of mutagens for *Rhizobium* spp. have been reported (Schwinghamer & Dalmas, 1961; Raina & Modi, 1969; Lorkiewicz *et al.*, 1971; Kaushik & Venkataraman, 1972*a, b*; Beringer, 1973; Meade & Signer, 1977), a comparison of results obtained with different mutagens is often difficult for a number of reasons. Firstly, the work has usually been carried out with different species or strains of *Rhizobium* under different experimental conditions. Secondly, the effectiveness of the mutagens has often been determined by detection of auxotrophs using a non-selective procedure which is essentially non-quantitative. Thirdly, spontaneous mutation frequencies, with which the induced frequencies must be compared, are rarely given.

This paper describes a comparative study to find the most effective mutagen for *Rhizobium trifolii*.

METHODS

Bacteria. *Rhizobium trifolii* P3 was obtained from Professor A. J. Holding (Department of Agricultural and Food Microbiology, Queen's University of Belfast, Northern Ireland); *R. trifolii* DC1t was a spontaneous rifampicin-resistant mutant and *R. trifolii* DC11t (*ade-1*) was an MNNG-induced auxotroph of *R. trifolii* P3. *Escherichia coli* 1830 (Beringer *et al.*, 1978) carried the plasmid pJB4JI containing Tn5 which confers kanamycin resistance.

Media. Complete medium (TY) and minimal medium (SY) for growth of *Rhizobium* spp. were as described by Beringer (1974). Media were solidified by addition of 1.5 % (w/v) Difco Bacto-agar.

Buffers. All buffers were made up in distilled water. Phosphate buffer, 0.067 M, pH 7.0, contained 4.56 g $\text{KH}_2\text{PO}_4 \text{ l}^{-1}$ and 4.75 g $\text{Na}_2\text{HPO}_4 \text{ l}^{-1}$. Acetate buffer, 0.1 M, pH 4.5, contained 4.02 g $\text{CH}_3\text{COONa l}^{-1}$. Tris/saline buffer, pH 7.6, contained 8.5 g NaCl l^{-1} in 0.2 M Tris.

† Present address: Department of Genetics, University of Birmingham, P.O. Box 363, Birmingham B15 2TT.

Chemicals. Ethyl methanesulphonate (EMS) and methyl methanesulphonate (MMS) (both from Eastman Kodak, Rochester, N.Y., U.S.A.) were stored in the dark at room temperature. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) was dissolved in phosphate buffer, pH 7.0, at a concentration of 1 mg ml⁻¹ and stored at -20 °C. Decarbamoyl mitomycin C (DCMTC; a gift from Kyowa Hakko Kogyo Co., Tokyo, Japan) was stored in phosphate buffer at a concentration of 100 µg ml⁻¹ at 4 °C. Rifampicin (Sigma) was dissolved in dimethyl sulphoxide and used at a final concentration of 25 µg ml⁻¹. All other chemicals were of standard analytical grade.

Mutagenesis. Exponential-phase cultures (approximately 2 × 10⁸ viable units ml⁻¹) were used in all mutagenesis experiments. Chemical mutagens were removed from 0.5 ml samples of the treated culture by centrifugation in a Quickfit (Corning, Stone, Staffs.) microcentrifuge and the bacteria were resuspended in TY medium; this centrifugation and resuspension procedure was repeated. Measurements of viability were made during the course of each experiment. The mutation frequency was measured following growth of the bacteria for a minimum of six generations in TY medium.

Mutagenic treatment. For treatment with MNNG, EMS, MMS and DCMTC, bacteria collected by centrifuging were washed and resuspended in 0.5 vol. phosphate buffer (unless otherwise stated), and an equal volume of phosphate buffer containing dissolved mutagen was added to give the desired final concentration. For treatment with nitrous acid, 9 ml culture was centrifuged and the bacteria were resuspended in 3 ml distilled water, to which was added 3 ml acetate buffer, pH 4.5, and 3 ml 0.01 M-NaNO₂ to start the reaction; the mutagenic action was stopped by a 100-fold dilution of the sample into TY broth. For ultraviolet (u.v.) irradiation, a culture was washed twice and resuspended in phosphate buffer, and a 5 ml volume was irradiated using an Hanovia model 12 germicidal lamp (Hanovia Lamps, Slough, Berks.) with an incident dose rate of 1.05 J m⁻² s⁻¹. Gamma-irradiation of bacteria was carried out in TY broth using a ⁶⁰Co source at a dose rate of 3.37 krad min⁻¹; oxygen was bubbled through the bacterial suspension during irradiation.

All mutagens were used at concentrations or doses which were lethal for a fraction of the population during the course of the experiment to ensure that damage was occurring, presumably to the DNA. In the case of nitrous acid, however, a preliminary experiment indicated that the low pH required for this experiment was in itself lethal for the rhizobia.

Transposon mutagenesis. The plasmid pJB4JI was transferred into *R. trifolii* DC1t by the membrane mating procedure described by Jacob *et al.* (1976) except that 0.1 ml volumes of donor were used and the membrane filter was of 25 mm diameter. Selection was for kanamycin resistance.

Choice of a suitable marker for the study of induction of mutation. Rifampicin resistance was chosen as a suitable marker for these experiments because it was readily selectable, no background growth of sensitive cells occurred, and in *R. trifolii* P3 its spontaneous mutation frequency was low (approximately 2 × 10⁻⁸), allowing high resolution studies of induced mutagenesis. An increase of fivefold or less in the mutation frequency was not considered significant since, because of variation and sampling error, the measured spontaneous mutation frequency could vary almost as much.

RESULTS

Induced mutation in R. trifolii P3

The results of treatment of *R. trifolii* P3 with three different mutagenic agents, two of which were effective (MNNG and u.v.) and one which was not (EMS), are given in Fig. 1. A summary of these results and those obtained with other mutagens is given in Table 1.

MNNG was the most effective mutagen, the maximum absolute mutation frequency for rifampicin resistance being 3 × 10⁻⁶. A similar result was obtained when the mutagenesis was carried out in TY broth. U.v. irradiation was the only other mutagenic agent of those tested found to be capable of mutating *R. trifolii* P3 to a significant extent. A maximum frequency of 8 × 10⁻⁷ rifampicin-resistant mutants was obtained, representing an approximately 40-fold increase over the spontaneous mutation frequency.

No evidence of any significant increase in the frequency of mutation to rifampicin resistance was found using other potentially mutagenic agents (EMS, MMS, DCMTC, nitrous acid and gamma rays). The inability of EMS, a highly effective mutagen for *Escherichia coli* B/r (Sweet & Moseley, 1976), to induce mutants in *R. trifolii* was rather surprising. The treatment with EMS was repeated using the conditions described by Meade & Signer (1977), by which they obtained a 300-fold increase in the frequency of rifampicin-resistant mutants of *R. meliloti*. There was no evidence of mutation in *R. trifolii* P3. EMS was also

Fig. 1. Effect of three different mutagenic agents on survival (●, ■) and mutation (○, □) of *R. trifolii* P3: (a) MNNG treatment ($75 \mu\text{g ml}^{-1}$) carried out in phosphate buffer (●, ○) or TY broth (■, □); (b) u.v. irradiation; (c) EMS treatment (0.25 %, w/v).

Table 1. *Lethal and mutagenic effects of a variety of common mutagenic agents on R. trifolii P3*

Mutagen	Lethality (D_{37})*	Mutagenesis (increase over spontaneous mutation frequency)†
MNNG	$60 \mu\text{g ml}^{-1} \text{ h}$	$\times 200$
MNNG (TY broth)	$29 \mu\text{g ml}^{-1} \text{ h}$	$\times 400$
U.v.	8.5 J m^{-2}	$\times 40$
EMS	$0.07 \% \text{ h}$	$\times 1$
MMS	$0.05 \% \text{ h}$	$\times 1$
DCMTC	$0.03 \mu\text{g ml}^{-1} \text{ h}$	$\times 1$
Nitrous acid	Not determined	$\times 1$
Gamma rays	2 krad	$\times 1$

* The D_{37} value is the dose required to reduce the viability of the original population to 37 % and is equivalent to that needed to kill a single bacterium. These values were calculated assuming the dose received is a function of the concentration of the mutagen used multiplied by the time of exposure.

† Mutation to rifampicin resistance: the spontaneous mutation frequency was approximately 2×10^{-8} .

unable to induce revertants of an adenine auxotroph of *R. trifolii* P3 (data not shown). Preliminary experiments have indicated that EMS is also an ineffective mutagen for *R. leguminosarum* 300 and *R. phaseoli* 8086 (M. Al-Doori, personal communication).

Transposon mutagenesis

The plasmid pJB4JI carries the transposon Tn5 which codes for kanamycin resistance. It is unstable in *Rhizobium* spp. because of the presence of the phage Mu genome which is also inserted in this plasmid (Boucher *et al.*, 1977; Beringer *et al.*, 1978). Thus, rhizobia which are kanamycin-resistant as a result of transfer of this plasmid from *E. coli* will almost certainly have the transposon inserted into either chromosomal or resident-plasmid DNA.

It is unlikely that rifampicin-resistant mutants would be obtained by transposon mutagenesis because the insertion event leads to a major disruption of the gene and is therefore unlikely to result in a viable phenotype. Thus, we decided to look for auxotrophic mutants which would be viable in a rich medium. This method was compared with MNNG mutagenesis.

An MNNG-treated culture which yielded rifampicin-resistant mutants at a frequency of 3×10^{-6} was screened for the presence of auxotrophs. Eight auxotrophs were isolated from 2718 colonies examined, a frequency of 0.3%. The requirements of two of these were identified: one required methionine and the other adenine or hypoxanthine. In a second similar experiment a further three auxotrophs were identified from 3261 colonies: one required cysteine, one methionine and one tryptophan.

In a cross between *E. coli* and *R. trifolii* DC1t in which the frequency of transfer of kanamycin resistance per recipient was 3.5×10^{-6} , a total of 4600 colonies were screened and 13 auxotrophs, a frequency of 0.3%, were isolated: two required histidine, two methionine, two adenine or hypoxanthine, one uracil, one cysteine, one tryptophan, and four were not identified.

DISCUSSION

The ability of only two out of seven mutagenic agents to cause significant mutation in *R. trifolii* demonstrates clearly that agents mutagenic for one organism (e.g. *E. coli*) are not necessarily mutagenic for others. The inability of EMS to induce mutations in *R. trifolii* P3 was rather surprising since this alkylating agent is a very efficient mutagen for *E. coli* B/r. However, *R. trifolii* P3 is considerably more sensitive to the lethal effects of EMS than *E. coli* B/r (cf. Sweet & Moseley, 1976) and so the lack of mutation may reflect an inability of *R. trifolii* P3 to tolerate and/or repair lethal DNA lesions caused by this mutagen.

The extent of mutation induced by MNNG and u.v. was not high, since induced mutation frequencies may be in excess of 1000-fold greater than the spontaneous mutation frequency (Sweet & Moseley, 1976). The reasons for this resistance to mutagenesis are not clear but the mechanism of mutation, i.e. repair-dependent or -independent (Drake & Baltz, 1976), and the pathways of repair available to the organism will influence the extent of induced mutation.

Transposon mutagenesis was as effective as MNNG mutagenesis in both the extent of induction of, and in the range of, auxotrophic mutations, suggesting that insertion of the transposon is fairly random (Beringer *et al.*, 1978), although preferred sites within short regions of DNA are likely to exist (Botstein & Kleckner, 1977).

This study indicates that it is worthwhile to test the efficacy of a variety of common mutagens on strains or species of *Rhizobium* or, indeed, other untested species of bacteria before undertaking a search for mutants. As regards *R. trifolii*, MNNG and u.v. are likely to be most useful when looking for mutants in which only minor alterations of the gene, e.g. point mutations, are likely to be tolerated. Transposition will only result in the production of mutants which are viable following a gross alteration in the mutated gene. It is, however, extremely useful for the production of mutants which would otherwise have a non-selectable phenotype, and has, for example, been used for the isolation and mapping of symbiotic mutants of *R. trifolii*, *R. meliloti* and *R. leguminosarum* (D. A. Walton & M. Al-Doori, unpublished; Meade *et al.*, 1979; Buchanan-Wollaston *et al.*, 1980).

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