SHORT COMMUNICATION

Isolation and Properties of an Ultraviolet-sensitive Mutant of *Rhizobium trifolii*

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In an attempt to isolate a strain of *Rhizobium trifolii* which could be highly mutated by ultraviolet light (u.v.), a u.v.-sensitive mutant was isolated using a semi-selective procedure. The mutant was not only 85 times more sensitive than the wild-type to the lethal effects of u.v., but was mutated at u.v. doses which had little mutagenic effect on the wild-type. Its sensitivity to the mutagenic agents methyl methanesulphonate and gamma rays was unaltered, but its spontaneous mutation frequencies for two antibiotic resistances were increased. The mutation conferring u.v. sensitivity was mapped on the chromosome of *Rhizobium leguminosarum* 300 in a position between the markers *ser-2* and *ade-88*. Unsuccessful attempts were made to transfer into the u.v.-sensitive mutant any one of a number of plasmids known to decrease the lethality of u.v. and enhance its mutagenicity.

INTRODUCTION

In a study of the ability of a variety of common mutagens to induce mutations in *Rhizobium trifolii*, only *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and ultraviolet light (u.v.) were found to be mutagenic (Walton & Moseley, 1981). The induced mutation frequencies obtained were not high and so an attempt was made to construct a strain of *R. trifolii* which was more susceptible to u.v.-induced mutagenesis.

Some u.v.-sensitive mutants of *Escherichia coli* are more susceptible than their respective wild-type parents to the mutagenic effects of u.v., mutations being produced at higher frequencies at low doses of u.v. (Witkin, 1966, 1967). These Uvr⁻ strains lack an endonuclease involved in the relatively error-free excision repair process (Braun & Grossman, 1974) so that a greater proportion of the pyrimidine dimers induced by u.v. are 'channelled' into the error-prone pathway of repair, resulting in an enhanced yield of mutants at low u.v. doses. A number of plasmids also increase the mutagenic effect of u.v. and at the same time reduce its lethal effect probably by contributing to, and thereby amplifying, the error-prone pathway of repair (Mortelmans & Stocker, 1976; Walker & Dobson, 1979).

This paper describes the isolation and some of the properties of a u.v.-sensitive mutant of *R. trifolii* and attempts to transfer into it any one of a number of plasmids known to enhance u.v. mutagenesis in other species.

METHODS

Bacteria and plasmids. *Rhizobium trifolii* P3 was obtained from Professor A. J. Holding (Department of Agricultural and Food Microbiology, Queen's University of Belfast, Northern Ireland). Derivatives of P3 used in this work were DC5t (*uvs-1*), DC6t (*uvs-1 rif-2*) and DC8t [*uvs-1 rif-2* (R68.45)]; the latter was obtained by

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transferring the plasmid R68.45 from *E. coli* J5-3(R68.45) (obtained from Dr J. E. Beringer, Soil Microbiology Department, Rothamsted Experimental Station, Herts.) into *R. trifolii* DC6t. Derivatives of *R. leguminosarum* 300 obtained from Dr J. E. Beringer were 1433 (*ade-27 rib-2 str-69*), 1628 (*ura-14 ade-27 str-75*), 1062 (*ura-14 trp-16 str-86*), 1056 (*ura-14 met-14 str-84*), 1783 (*ser-2 met/cys-19*), 1784 (*ser-2 ade-88*) and 1629 (*cys-8 ura-14 ade-88 str-69*); *R. leguminosarum* DC11 and DC21 were spontaneous streptomycin-resistant derivatives of strains 1783 and 1784, respectively. Other species used were *E. coli* W677 [*thr leu thi lac* (R751-SU2)], *Pseudomonas aeruginosa* PU21 [FP-*ilvB112 leu-1 str-1 rif*] carrying plasmid pMG1, R931 or R2, *Pseudomonas putida* AC34 [*ade* (R751-SU2)] and *Salmonella typhimurium* TA100 [*his bio Δuvr* (pKM101)] (McCann *et al.*, 1975): all these strains, except *S. typhimurium* TA100, were obtained from Dr G. A. Jacoby (Massachusetts General Hospital, Boston, Mass., U.S.A.). The plasmid phenotypes were as described in Bukhari *et al.* (1977).

Bacteriophage. A bacteriophage infective for *R. trifolii* P3 and designated Rt1 was isolated from soil. A 5 g sample of soil was shaken in 20 ml phosphate buffer and allowed to settle overnight. A sample of the supernatant was sterilized by passage through a Millipore filter (0.45 µm pore size) and 1 ml was added to a 10 ml exponential-phase culture of *R. trifolii* P3. Following overnight incubation, the culture was centrifuged to remove bacteria and dilutions of the supernatant were plated by the soft-agar layer method (Adams, 1959) to obtain plaques. The virus was purified by stabbing an individual plaque, suspending in 1 ml TY broth, then replating to obtain single plaques. This procedure was carried out twice.

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

Buffer. Phosphate buffer, 0.067 M, pH 7.0, contained 4.56 g KH_2PO_4 and 4.75 g Na_2HPO_4 in 1 litre distilled water.

U.v. irradiation, gamma irradiation and methyl methanesulphonate (MMS) treatment. These were carried out as described in Walton & Moseley (1981). The lethality of these mutagenic agents was measured and D_{37} values were calculated from the survival curves. The D_{37} value is the dose required to reduce the viability of the original population to 37% and is equivalent to that required to kill a single bacterium.

Isolation of a u.v.-sensitive mutant of *R. trifolii* P3. The semi-selective method used was based on that of Howard-Flanders & Theriot (1962). A high-titre lysate of phage Rt1 was centrifuged at 38000 g for 2 h and resuspended in an equal volume of distilled water (1.3×10^{10} plaque-forming units ml^{-1}). The lysate was exposed to a u.v. dose of 70 J m^{-2} (13% survival), equilibrated at 30 °C and an equal volume of exponential-phase bacteria (7.0×10^7 viable units ml^{-1}) was added. Adsorption was allowed to take place for 25 min, giving an approximate multiplicity of infection of 10 (i.e. for viable and non-viable phage), and about 1000 bacteria were spread per plate. After incubation for 3 d, surviving colonies were streaked across TY plates and successive sections were exposed to u.v. doses of 0, 21, 42 and 63 J m^{-2} to identify u.v.-sensitive clones.

Assessment of mutation frequencies. Resistance to rifampicin (25 µg ml^{-1} ; Sigma) was chosen for the measurement of spontaneous and u.v.-induced mutation frequencies for reasons described elsewhere (Walton & Moseley, 1981). Spontaneous resistance to kanamycin (50 µg ml^{-1} ; Sigma) was also measured.

Transfer of plasmids. Plasmids were transferred by conjugation using 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.

Mapping. Mapping of the *uvs* marker was carried out in *R. leguminosarum* 300 by R68.45-mediated recombination since suitable recipients of this strain exist and a genetic map has been published (Beringer *et al.*, 1978). A series of crosses was carried out with seven recipient strains of *R. leguminosarum*: 1433, 1628, 1062, 1056, 1629, DC11 and DC21. Each strain carried two genetic markers flanking a section of the bacterial chromosome, the seven sections covering a large part of the chromosome. Crosses were not carried out with two other strains which carried markers flanking the remainder of the chromosome. When *Rhizobium* receives a section of the chromosome flanked by two genetic markers, a high proportion of the recipients also receive any marker within that section (Beringer *et al.*, 1978). Thus, selection was made for the transfer of pairs of donor alleles and the recipient bacteria were tested for co-inheritance of the *uvs* marker. Once the relevant section carrying the *uvs* marker had been identified, two-point crosses were carried out to locate the marker more accurately.

RESULTS

Isolation of a u.v.-sensitive mutant

The principle of this isolation procedure was based on the finding that the u.v.-sensitive strains of *E. coli* have a considerably reduced ability to repair u.v. damage in irradiated phage for which they are host (Ellison *et al.*, 1960; Witkin, 1967). Thus, survivors from a population

Fig. 1. Efficiency of plating of u.v.-irradiated phage Rt1 on *R. trifolii* P3 (●) and *R. trifolii* DC5t (▲). The efficiencies of plating are expressed relative to the efficiency of plating of unirradiated phage on the wild-type strain P3.

Fig. 2. Effect of u.v. irradiation on survival (●, ■) and mutation (○, □) of *R. trifolii* P3 (●, ○) and *R. trifolii* DC5t (■, □).

of bacteria infected with irradiated phage may include u.v.-sensitive mutants (Howard-Flanders & Theriot, 1962). In the experiment described here, one u.v.-sensitive mutant, designated DC5t, was isolated from among 301 survivors. Confirmation that the mutant was indeed a product of the isolation procedure was obtained from an experiment in which unirradiated and u.v.-irradiated phage were plated on *R. trifolii* P3 or DC5t. Unirradiated phage plated with equal efficiency on both strains but irradiated phage had a reduced efficiency of plating on DC5t relative to P3 (Fig. 1).

Properties of the u.v.-sensitive mutant

The extreme sensitivity of *R. trifolii* mutant DC5t to u.v. can be seen from Fig. 2(a); the D_{37} values for *R. trifolii* P3 and DC5t were 8.5 and 0.1 J m⁻², respectively, i.e. mutant DC5t was 85 times more sensitive than its parental strain to the lethal effects of u.v. Mutant DC5t was not more sensitive than its parental strain to the lethal effects of MMS and gamma rays, agents which are capable of indirectly or directly causing single-strand breaks in DNA. The

D_{37} values for *R. trifolii* P3 treated with MMS or gamma rays were 0.05 % h and 2.1 krad, respectively, and for DC5t, 0.06 % h and 2.6 krad, respectively.

The results of a u.v. mutagenesis experiment on *R. trifolii* P3 and DC5t are shown in Fig. 2(b). Induced mutation to rifampicin resistance in *R. trifolii* DC5t was observed at extremely low u.v. doses where virtually no induction of mutation in the wild-type occurred. The increase over the spontaneous mutation frequency in DC5t was only about fivefold. However, the spontaneous mutation frequency in DC5t was about six times higher than that for the parental wild-type, so that the maximum induced frequencies for DC5t were 1×10^{-6} and for P3, 8×10^{-7} (data not shown). A fivefold increase in the spontaneous mutation frequency for kanamycin resistance was also observed.

Mutant DC5t formed normal numbers of nitrogen-fixing nodules on clover plants (*Trifolium repens* cv. Huia).

Mapping of the *uvr* marker

No co-inheritance of the *uvr* marker with the regions flanked by the *rib-2⁺/ade-27⁺*, *ade-27⁺/ura-14⁺*, *ura-14⁺/trp-16⁺*, *ura-14⁺/met-14⁺* or *ade-88⁺/cys-8⁺* markers was found. However, it was 15 % co-inherited with the region flanked by *met/cys-19⁺* and *ser-2⁺* and 65 % co-inherited with the region flanked by *ser-2⁺* and *ade-88⁺*. When selection was made for transfer of either *ser-2⁺* or *ade-88⁺*, the *uvr* marker was 24 % co-inherited with *ade-88⁺* and 18 % co-inherited with *ser-2⁺*. Since *ade-88⁺* and *ser-2⁺* showed 3 % co-inheritance, the linkage data are consistent with a position for the *uvr* marker between *ade-88* and *ser-2* on the genetic map of *R. leguminosarum* 300.

Attempts to transfer into *R. trifolii* DC6t plasmids known to enhance mutagenesis

One of the factors limiting the maximum u.v.-induced mutation frequency for *R. trifolii* DC5t was its extreme sensitivity to the lethal effects of u.v. radiation. Conjugational transfer into *R. trifolii* DC6t (a rifampicin-resistant derivative of DC5t) was attempted for each of five plasmids known to enhance u.v. mutagenesis and survival. In such experiments there was no evidence of transfer of pKM101 from *S. typhimurium* TA100 (selection for ampicillin resistance), of pMG1, R931 or R2 from *P. aeruginosa* PU21 (selection for streptomycin resistance) or of R751-SU2 from *E. coli* W677 or *P. putida* AC34 (selection for streptomycin resistance).

DISCUSSION

The mutant *R. trifolii* DC5t was 85 times more sensitive than its parental strain to the lethal effects of u.v., a figure comparable to the relative sensitivity of a *uvrA* strain of *E. coli* which was 60 times more sensitive than the wild-type (Howard-Flanders & Boyce, 1966). Mutant DC5t was unable to host-cell reactivate irradiated phage, as predicted by the method of isolation, and was not significantly more sensitive than its parental strain to the lethal effects of MMS and gamma rays, suggesting that the impaired repair function in this strain was fairly specific for u.v. damage. However, the spontaneous mutation frequency for at least two markers was enhanced in *R. trifolii* DC5t, suggesting that the missing function may be involved in the repair of at least some damage introduced spontaneously into the DNA.

U.v.-induced mutation in this strain occurred at very low doses which had little effect on the wild-type parental strain either with respect to its mutagenic or lethal response. This has also been observed in *Uvr⁻* strains of *E. coli* (Witkin, 1966; Bridges & Munson, 1966).

The lack of inheritance of plasmids pKM101, pMG1, R931 and R2 in *R. trifolii* was perhaps not surprising since there are no known reports of establishment in *Rhizobium* of plasmids other than those belonging to the incompatibility group P1. However, the plasmid R751-SU2, which is classified in the incompatibility group P1 (Hedges & Jacob, 1975; Bukhari *et al.*, 1977), was not transmissible to *R. trifolii*. This may be because this hybrid

plasmid does not have the broad host range properties of group P1, e.g. this plasmid is also not transferable to *P. aeruginosa* (G. A. Jacoby, personal communication), or because the selected plasmid marker was not expressed.

From a practical point of view, the maximum u.v.-induced frequencies of rifampicin-resistant mutants were similar for both *R. trifolii* DC5t and the wild-type strain suggesting that there was no particular advantage in using the former strain for the isolation of mutants.

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