SHORT COMMUNICATION

Ultraviolet Sensitivity and Mutagenesis of *Azotobacter*

By BETTY E. TERZAGHI

*Plant Physiology Division, Department of Scientific and Industrial Research,
Private Bag, Palmerston North, New Zealand*

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Survival of *Azotobacter vinelandii* and *Azomonas agilis* after ultraviolet irradiation was consistent with single-hit inactivation of DNA. Mutants of *Azotobacter vinelandii* were generated, but all continued to segregate after two or more purifications.

INTRODUCTION

Metabolic mutants of *Azotobacter vinelandii* were first isolated over 30 years ago (Karlsson & Barker, 1948; Wyss & Wyss, 1950), but subsequent mutant loss or instability, coupled with reports of failures to mutate successfully (Mishra & Wyss, 1968), have contributed to a shift in emphasis in nitrogen fixation research from *Azotobacter* to the agronomically unimportant but more readily manipulated *Klebsiella*. However, it has not been clear whether *Azotobacter* is difficult to mutate *per se*, or whether selection procedures have been inadequate. Recently, Sadoff *et al.* (1979) argued that *Azotobacter vinelandii* contains 40 chromosomes per cell and therefore failure to isolate mutants could result from difficulties in segregation rather than mutagenesis. Stable nitrogen fixation (Nif) mutants (Wyss & Wyss, 1950; Green *et al.*, 1953; Fisher & Brill, 1969) and drug resistance mutants (Page & Sadoff, 1976; Bishop & Brill, 1977) have been readily isolated using selective media. Recently, Leach & Battikhi (1978) have isolated amino acid and vitamin auxotrophs and drug resistance mutants of *Azotobacter vinelandii* by use of a technique which breaks up cell duplexes prior to mutagenesis, thereby allowing direct selection of single mutant cells. Results on the stability of these strains have not yet been reported.

Our aim was to isolate nitrogenase-derepressed mutants of *Azotobacter* (Terzaghi, 1980) which resulted from single mutational events. Ultraviolet (u.v.) mutagenesis was chosen as the most easily controllable method of treatment. In this paper, I present results on the u.v. sensitivity of *Azotobacter vinelandii* and *Azomonas agilis* and suggest that difficulties in obtaining mutants reside in genetic instability rather than in mutagenesis.

METHODS

Bacteria. *Azotobacter vinelandii* strain UW was obtained from W. Brill and *Azomonas agilis* A30 from E. Mulder.

Media. Modified Burk's medium (B medium) contained (g l⁻¹ in distilled water): MgSO₄·7H₂O, 0·2; FeSO₄·7H₂O, 0·005; Na₂MoO₄·2H₂O, 0·00024; KH₂PO₄, 0·2; K₂HPO₄, 0·8; glucose, 5; CaCl₂·2H₂O, 0·0085 (added after sterilization and cooling). B:Ac medium was B medium supplemented after autoclaving with filter-sterilized ammonium acetate to a final concentration of 0·1% (w/v). Tetrazolium agar and eosin/methylene blue (EMB) agar were prepared in B:Ac agar containing 2% (w/v) glucose, using methods described by Miller (1972). With tetrazolium, it was essential to use fresh medium to obtain reproducible colour formation. For BB agar, separate sterilized 0·2% (w/v) solutions of bromocresol purple and bromthymol blue were added in equal amounts (each to a final concentration of 0·001%, w/v) to B:Ac agar con-

Fig. 1. Survival of *Azotobacter vinelandii* and *Azomonas agilis* following increasing exposure to u.v. as determined by colony formation on B:Ac medium at 28 °C. Bacteria were grown in calcium-free medium, suspended in 0.1 M-MgSO₄ and irradiated in 10 ml quantities in 9 cm glass Petri dishes under conditions where 8 s exposure approximated to a dose of 10 J m⁻². ○, *Azomonas agilis* strain A30; □, *Azotobacter vinelandii* strain UW grown in B:Ac medium; ■, *Azotobacter vinelandii* strain UW grown in B medium.

taining 2% (w/v) glucose. All solid media contained 1.5% (w/v) agar (Difco-Bacto or Davis New Zealand). When required, the pH was adjusted by adding 0.1 M KOH or HCl prior to sterilization.

Growth conditions. Bacteria were grown by inoculating from slants into Erlenmeyer flasks containing B medium at 20% of the flask volume and gently swirling (50 rev. min⁻¹) at 25 °C until growth ceased. All experiments were performed by subculturing (1%, v/v) from such freshly grown cultures into the appropriate media.

U.v. mutagenesis. Strains UW and A30 were subcultured from medium B into calcium-free B:Ac medium and grown to an A_{660} of 0.2 (5.6×10^7 colony-forming units ml⁻¹). The cultures were chilled, centrifuged, washed in 0.1 M-MgSO₄, resuspended at half the original cell concentration in 0.1 M-MgSO₄, and u.v.-irradiated in 9 cm glass Petri dishes for varying times in 10 ml quantities with protection from photoreactivation. Samples were plated directly on B:Ac agar to determine survival. The irradiated cultures were centrifuged, resuspended and grown in both B and B:Ac media at 25 °C until growth ceased and then plated on all three indicator media. A u.v. survival curve was also determined for strain UW grown in calcium-free B medium.

Mutant selection and purification. Indicator plates were incubated for 5 d at 28 °C and then examined, under magnification, for aberrant colony colour. Large colonies or sectors were stabbed with a fine wire for resuspension in B:Ac medium and direct restreaking on indicator media. This process was repeated a minimum of four times or until the restreaked culture showed no further segregation.

RESULTS

U.v. sensitivity. The survival curves after u.v. irradiation (Fig. 1) show that for both species sensitivity to u.v. dose over several orders of magnitude was consistent with that reported for other bacteria (Hopwood, 1970). The species differed principally in that the curve for strain UW had an initial shoulder in contrast to the single-hit response of strain A30. The rate of inactivation of strain UW grown in B:Ac medium was faster than for strain UW grown in B medium.

Mutant screening. Only strain UW was examined for mutant production following u.v. irradiation. On all indicator media, the frequency of abnormally coloured colonies increased with increased exposure to u.v., as did the frequency of small colonies and colonies with 'nibbled' edges. Many differently coloured colonies were observed, ranging from white to

rose to intense blue on EMB agar, grey to gold on BB agar, and speckled to uniformly red on tetrazolium agar. Not all isolates picked as a mutant on one indicator showed a mutant phenotype on the other indicators.

Mutant characterization. All of 30 purified mutants were Nif^+ as determined by their ability to grow aerobically in liquid medium B. Four isolates gave positive acetylene reduction activity on nitrogen-supplemented media and are further characterized in a separate communication (Terzaghi, 1980). The remaining isolates have not been further characterized.

DISCUSSION

No difficulties were encountered in either generating or selecting mutants despite the fact that no deliberate attempts were made to break cell duplexes. However, it is possible that single cells, which Leach & Battikhi (1978) purposefully generated, were inadvertently produced here during growth in calcium-free medium prior to mutagenesis. Microscopic examination of the cultures showed many single cells, but no systematic study was made of this point. Similarly, the dose-response to u.v. (Fig. 1) indicated a rate of inactivation for both strains grown in B:Ac medium that was comparable to the rate for *Escherichia coli* (Miller, 1972). Although bacteria grown on N_2 were more resistant to u.v., neither growth condition gave an increased rate of survival consistent with having 10 times more DNA than *E. coli* (Sadoff *et al.*, 1971). Therefore, if an *Azotobacter vinelandii* cell contains 40 chromosomes (Sadoff *et al.*, 1979), and its u.v. sensitive target size and single chromosome size (Sadoff *et al.*, 1979) are equivalent to that of *E. coli*, most of the DNA must be in multiple copies that are not biologically functional.

The problems of segregation and mutant stability remained uncontrolled in this study. Of the 30 isolates, not one was phenotypically homogeneous after the second purification and in those isolates which continued to produce sectorized colonies, only the segregants with wild-type phenotype were stable. In most cases, however, multiple repurification eventually led to non-segregating mutants. Thus the problems in obtaining *Azotobacter* mutants appear to reside, not in mutagenesis, but in segregation or stabilization of mutated DNA.

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