Isolation and Physiological Characterization of Mitomycin C-sensitive/ UV-sensitive Mutants in *Bacteroides fragilis*

By VALERIE R. ABRATT, DAVID T. JONES AND
DAVID R. WOODS*

CSIR Microbial Genetics Research Unit, Department of Microbiology, University of Cape Town,
Rondebosch 7700, South Africa

(Received 23 October 1984; revised 25 March 1985)

Mutants of *Bacteroides fragilis* sensitive to mitomycin C were isolated after mutagenesis with ethyl methane sulphonate. One mutant (MTC25) was markedly sensitive to mitomycin C but was unaffected as regards UV sensitivity; another mutant (UVS9) was sensitive to UV radiation but was only moderately sensitive to mitomycin C. Caffeine decreased the survival after UV-irradiation of the wild-type, MTC25 and UVS9 strains by the same relative amount. Aerobic liquid holding recovery occurred in each of the three strains. The MTC25 and UVS9 mutants showed reduced host cell phage reactivation. The wild-type, MTC25 and UVS9 strains all showed UV- and H₂O₂-induced phage reactivation. The physiological characterization of the MTC25 and UVS9 mutants indicates that it is possible to differentiate between mechanisms for the repair of mitomycin C- and UV-induced DNA damage in *B. fragilis*.

INTRODUCTION

Although DNA repair mechanisms have been extensively studied in *Escherichia coli* and other aerobic bacteria, the DNA repair systems of obligate anaerobes have been largely neglected. The anaerobe *Bacteroides fragilis* is interesting in that survival after irradiation with far-UV light is affected by oxygen (Jones *et al.*, 1980; Slade *et al.*, 1981). Furthermore, novel O₂- and H₂O₂-induced phage reactivation systems have been reported for *B. fragilis* (Slade *et al.*, 1983*a, b*). Since UV-sensitive mutants have played a major role in the elucidation of DNA repair mechanisms in *E. coli* we attempted the isolation of UV-sensitive mutants of *B. fragilis*.

Mutants of *B. fragilis* and other obligate anaerobes are difficult to obtain (Sebald & Costilow, 1975; Van Tassel & Wilkins, 1978; Droffner & Yamamoto, 1983); indeed, Droffner & Yamamoto (1983) reported the inhibition of mutagenesis in *Salmonella typhimurium* by anaerobic conditions. Since a correlation between UV sensitivity and mitomycin C sensitivity has been reported in *E. coli* (Boyce & Howard-Flanders, 1964), *Micrococcus radiodurans* (Moseley, 1967), *Bacillus subtilis* (Okubo & Romig, 1966) and *S. typhimurium* (Ames *et al.*, 1973), an indirect selection procedure utilizing mitomycin C was investigated. Mitomycin C has been reported to be chemically activated by reduction with hydrogen in the presence of a palladium catalyst, but it is highly unstable in this form and rapidly loses its DNA crosslinking ability (Szybalski & Iyer, 1967). Its suitability as a selective agent under anaerobic conditions had to be established.

METHODS

Bacteria and phages. These studies were done with a *B. fragilis* strain (Bf-2) and the phage b-1 which have been used in previous studies on far-UV irradiation effects (Jones *et al.*, 1980; Jones & Woods, 1981).

Media. Brain heart infusion broth and agar supplemented with haemin, menadione and cysteine (Holderman & Moore, 1972) were used for bacterial growth at 37 °C. Anaerobic one-quarter-strength Ringer's solution was used as a dilution buffer. All anaerobic manipulations were done under stringent anaerobic conditions in an anaerobic glove cabinet (Forma Scientific, Marietta, Ohio, USA) in an atmosphere of 75% N₂, 20% CO₂ and 5% H₂.

Mutagenesis and isolation of mutants. Ethyl methane sulphonate (2%, v/v) was added to exponential phase *B. fragilis* cells (OD_{600} 0.3) and the culture was incubated for 25 min at 37 °C (approximately 10% survival). The cells were sedimented by centrifugation, resuspended in fresh brain heart infusion broth and incubated for 20 h. The culture was diluted and plated to give approximately 100 c.f.u. per plate. After 18–20 h incubation the pin-prick-sized colonies were replica plated onto agar plates containing mitomycin C ($0.1 \mu\text{g ml}^{-1}$) and control plates without mitomycin C. Colonies which did not grow on the mitomycin C plates were retested for mitomycin C and UV sensitivity.

UV irradiation experiments. Methods for measuring survival after UV irradiation, liquid holding recovery, uninduced and UV- or H_2O_2 -induced reactivations of phage b-1 were as described by Jones & Woods (1981), Slade *et al.* (1983*a, b*) and Schumann *et al.* (1984).

RESULTS

Activity of mitomycin C under anaerobic conditions

The suitability of an indirect selection procedure for UV sensitivity based on the use of mitomycin C was investigated, as mitomycin C has been shown to be chemically activated to an unstable form under reducing conditions (Szybalski & Iyer, 1967). The MIC for *B. fragilis* Bf-2 cells ($100 \text{ c.f.u. ml}^{-1}$) plated on freshly poured plates placed immediately under anaerobic conditions, or plates stored aerobically for 24 h before exposure to anaerobic conditions in the anaerobic cabinet, was $0.14 \mu\text{g mitomycin C ml}^{-1}$. If plates containing mitomycin C were kept in anaerobic conditions for 24 h the compound lost activity and the MIC was greater than $7 \mu\text{g mitomycin C ml}^{-1}$.

Isolation of mitomycin C- and UV-sensitive mutants

After ethyl methane sulphonate mutagenesis, six mutants were identified as being sensitive to mitomycin C. Five of these showed similar sensitivity to mitomycin C (MIC $0.06 \mu\text{g ml}^{-1}$) and all showed wild-type resistance to UV. These strains may have been siblings and one of them (MTC25) was chosen for further study. Growth of the sixth mitomycin C-sensitive mutant was inhibited by $0.08 \mu\text{g mitomycin C ml}^{-1}$, a concentration intermediate between that which inhibited the MTC25 and wild-type strains. This mutant designated UVS9, showed increased sensitivity to UV radiation. Representative survival curves showing sensitivity to mitomycin C and UV radiation are shown in Figs 1 and 2. The mean UV radiation lethal dose (Harm, 1980) for the wild-type and MTC25 strains was 15 J m^{-2} (Table 1), and the relative sensitivity of the UVS9 mutant was 1.5-fold.

Effect of caffeine on survival after UV irradiation

The MIC of caffeine for the wild-type, MTC25 and UVS9 strains was 2.5 mg ml^{-1} . When these three strains were irradiated and plated on brain heart infusion agar containing $1 \text{ mg caffeine ml}^{-1}$, a marked decrease in survival was observed compared with the survival on the control plates (Fig. 2). The dose reduction factor (Harm, 1980) at 1% survival was 0.7 for each of the three strains.

Liquid holding recovery

The survival of irradiated stationary phase wild-type, MTC25 and UVS9 cells was enhanced after holding in aerobic one-quarter-strength Ringer's solution (Table 1). Unirradiated cells held in the aerobic Ringer's solution showed no change in survival. Irradiated wild-type and MTC25 strains held under anaerobic conditions showed a decrease in survival whereas the UVS9 strain showed a small increase in survival. Unirradiated cells held anaerobically showed no change. Jones & Woods (1981) reported that exponential phase cells showed higher liquid holding recovery levels than stationary phase cells and similar results were obtained with the wild-type, MTC25 and UVS9 strains.

Fig. 1

Fig. 2

Fig. 1. Effect of mitomycin C on the survival of *B. fragilis* strains. Stationary phase cultures were diluted 10^{-3} into anaerobic one-quarter-strength Ringer's solution containing $20 \mu\text{g}$ mitomycin C ml^{-1} and the number of c.f.u. determined. ■, Wild-type strain; ▲, strain MTC25; ●, strain UVS9.

Fig. 2. Effect of caffeine on the survival of UV-irradiated *B. fragilis* strains. Cells were irradiated with increasing doses of UV radiation (254 nm) and plated on brain heart infusion agar with and without 1 mg caffeine ml^{-1} . Wild-type strain plated with (□) and without (■) caffeine; strain MTC25 with (△) and without (▲) caffeine; strain UVS9 with (○) and without (●) caffeine.

Table 1. Repair phenotypes of wild-type *B. fragilis* and mutant strains

Strain	$*F_{0.37}$ (J m^{-2})	Mitomycin C MIC ($\mu\text{g ml}^{-1}$)	LHR†	
			Aerobic	Anaerobic
Wild-type	15	0.14	2.1	0.45
MTC25	15	0.06	1.8	0.48
UVS9	10	0.08	2.3	1.6

* Mean lethal dose (dose resulting in 37% survival of cells).

† Liquid holding recovery. Cells were irradiated to 1% survival and held at 30°C for 2 h in minimal salts solution (anaerobic) or one-quarter-strength Ringer's solution (aerobic). Results are expressed relative to irradiated cells plated immediately. Data from one of five independent experiments are given. The results from all the experiments were within $\pm 9\%$ of the values given.

Phage reactivation

Slade *et al.* (1983*a, b*) investigated the optimum conditions for UV- and H_2O_2 -induced phage reactivation in *B. fragilis* Bf-2. Similar experiments with the mutants MTC25 and UVS9 indicated that the optimum conditions of UV-irradiation and H_2O_2 concentration for phage reactivation were the same as for the wild-type. The efficiency of plating of the unirradiated phage on the wild-type, MTC25 and UVS9 strains was identical. The MTC25 and UVS9 mutants both showed a decrease in uninduced host cell phage reactivation and UV-induced phage reactivation relative to the wild-type (Table 2). The UVS9 mutant differed from the wild-type and MTC25 strains in that it showed a reduction in H_2O_2 -induced phage reactivation to about 33% of that seen in these strains.

Table 2. *Bacteriophage reactivation*

Phage b-1 was UV-irradiated (300 J m^{-2}), and the extent of uninduced host cell reactivation and UV- and H_2O_2 -induced host cell reactivation were determined as described previously (Slade *et al.*, 1983*a, b*).

Strain	Host cell reactivation		
	Uninduced*	Induced†	
		H_2O_2	UV
Wild-type	1	6.3 (± 2.8)	4.7 (± 0.7)
MTC25	0.22 (± 0.14)	6.7 (± 2.4)	2.6 (± 0.9)
UVS9	0.25 (± 0.11)	2.2 (± 1.9)	3.5 (± 0.6)

* Relative to the wild type expressed as 1. The mean of eight experiments is shown, $\pm$ the range.

† Relative to the uninduced level of that strain expressed as 1. The mean of six and four experiments is shown for H_2O_2 and UV, respectively, $\pm$ the range.

DISCUSSION

Although the activity of mitomycin C has been reported to be affected by anaerobic conditions (Szybalski & Iyer, 1967) it was successfully used for the isolation of two different mitomycin C-sensitive *B. fragilis* mutants by the use of controlled experimental conditions. The MTC25 mutant is markedly sensitive to mitomycin C but is unaffected as regards UV sensitivity. The UVS9 mutant is sensitive to UV but is only moderately sensitive to mitomycin C. A similar correlation between the repair of mitomycin C- and UV-induced lesions has been observed in *M. radiodurans* (Moseley & Copeland, 1978). In *M. radiodurans* it has been shown that two separate DNA excision repair pathways exist, one requiring a functional *mtcA* gene and the other functional *uvrC*, *uvrD* and *uvrE* genes, and that mutational blocks in both must exist for an excisionless phenotype to be expressed (Evans & Moseley, 1983; Moseley & Evans, 1983).

The *B. fragilis* UVS9 mutant showed a dose reduction factor (Harm, 1980) of 0.6 at 1% survival compared to the wild-type. The main difference between the UV survival curves of the wild-type and UVS9 strains was in the shoulder region where repair of the DNA damage occurs at low UV doses.

In *E. coli*, liquid holding recovery is observed in cells having functional *uvr* and *polA* gene products, but only if they have some other decreased repair capacity as is found in *E. coli* B or *E. coli* K12 *recA* mutants (Ganesan & Smith, 1968, 1969). In *B. fragilis*, aerobic liquid holding recovery occurs in the wild-type as well as in both mutant strains. Under anaerobic conditions, where the wild-type is functioning optimally, a negative liquid holding effect was observed. This is a phenomenon usually found in cells which are repair proficient and where holding is detrimental to recovery (Harm, 1980). The MTC25 mutant also showed a negative liquid holding effect, which is in agreement with the wild-type level of UV resistance found in this strain. The UV-sensitive UVS9 strains however, showed a tendency towards recovery on holding under anaerobic conditions, which correlates with its UV-sensitive phenotype. Jones & Woods (1981) reported the inhibition of liquid holding recovery under anaerobic conditions but did not observe the negative liquid holding effect.

H_2O_2 -induced phage reactivation revealed a further difference between the two *B. fragilis* mutant strains. Phage reactivation in the MTC25 mutant was induced to the same extent as in the wild-type by H_2O_2 , but the UVS9 mutant showed reduced H_2O_2 -induced reactivation.

The isolation and physiological characterization of the mitomycin C-sensitive and UV-sensitive mutants in *B. fragilis*, which is difficult to mutate, is an important and essential requirement for future studies on repair pathways in this important obligate anaerobe.

REFERENCES

- AMES, B. N., LEE, F. D. & DURSTAN, W. C. (1973). An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 782-786.
- BOYCE, R. P. & HOWARD-FLANDERS, P. (1964). Genetic control of DNA breakdown and repair in *E. coli* K12 treated with mitomycin C or ultraviolet light. *Zeitschrift für Vererbungslehre* **95**, 345-350.
- DROFFNER, M. L. & YAMAMOTO, N. (1983). Anaerobic cultures of *Salmonella typhimurium* do not exhibit inducible proteolytic function of the *recA* gene and *recBC* function. *Journal of Bacteriology* **156**, 962-965.
- EVANS, D. M. & MOSELEY, B. E. B. (1983). Roles of the *uvrC*, *uvrD*, *uvrE* and *mtcA* genes in the two pyrimidine dimer excision repair pathways of *Deinococcus radiodurans*. *Journal of Bacteriology* **156**, 576-583.
- GANESAN, A. K. & SMITH, K. C. (1968). Dark recovery processes in *E. coli* irradiated with UV light. I. Effect of *rec*⁻ mutants on liquid holding recovery. *Journal of Bacteriology* **96**, 365-373.
- GANESAN, A. K. & SMITH, K. C. (1969). Dark recovery processes in *E. coli* irradiated with UV light. II. Effects of *uvr* genes on liquid holding recovery. *Journal of Bacteriology* **97**, 1129-1133.
- HARM, W. (1980). *Biological Effects of Ultraviolet Radiation*. Cambridge: Cambridge University Press.
- HOLDERMAN, L. V. & MOORE, W. E. C. (editors) (1972). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg, Va.: Virginia Polytechnic Institute and State University Anaerobe Laboratory.
- JONES, D. T. & WOODS, D. R. (1981). Effect of oxygen on liquid holding recovery of *Bacteroides fragilis*. *Journal of Bacteriology* **145**, 1-7.
- JONES, D. T., ROBB, F. T. & WOODS, D. R. (1980). Effect of oxygen on *B. fragilis* survival after UV irradiation. *Journal of Bacteriology* **144**, 1179-1181.
- MOSELEY, B. E. B. (1967). The isolation and some properties of radiation-sensitive mutants of *Micrococcus radiodurans*. *Journal of General Microbiology* **49**, 293-300.
- MOSELEY, B. E. B. & COPELAND, H. J. R. (1978). Four mutants of *Micrococcus radiodurans* defective in the ability to repair DNA damaged by mitomycin C, two of which have wild type resistance to ultraviolet radiation. *Molecular and General Genetics* **160**, 331-337.
- MOSELEY, B. E. B. & EVANS, D. M. (1983). Isolation and properties of strains of *Micrococcus (Deinococcus) radiodurans* unable to excise ultraviolet light-induced pyrimidine dimers from DNA: evidence for two excision pathways. *Journal of General Microbiology* **129**, 2437-2445.
- OKUBO, S. & ROMIG, W. R. (1966). Impaired transformability of *B. subtilis* mutant sensitive to mitomycin C and ultraviolet radiation. *Journal of Molecular Biology* **15**, 440-454.
- SCHUMANN, J. P., JONES, D. T. & WOODS, D. R. (1984). Effect of UV irradiation on macromolecular synthesis and colony formation in *B. fragilis*. *Journal of General Microbiology* **130**, 771-777.
- SEBALD, M. & COSTILOW, R. N. (1975). Minimal growth requirements for *Clostridium perfringens* and isolation of auxotrophic mutants. *Applied Microbiology* **29**, 1-6.
- SLADE, H. J. K., JONES, D. T. & WOODS, D. R. (1981). Effect of oxygen radicals and peroxide on survival after U.V. irradiation and liquid holding recovery of *Bacteroides fragilis*. *Journal of Bacteriology* **147**, 685-687.
- SLADE, H. J. K., SCHUMANN, J. P., JONES, D. T. & WOODS, D. R. (1983a). Peroxide inducible phage reactivation in *B. fragilis*. *FEMS Microbiology Letters* **20**, 401-405.
- SLADE, H. J. K., SCHUMANN, J. P. S., PARKER, J. R., JONES, D. T. & WOODS, D. R. (1983b). Effect of oxygen on host cell reactivation in *B. fragilis*. *Journal of Bacteriology* **153**, 1545-1547.
- SZYBALSKI, W. & IYER, V. N. (1967). The mitomycins and porfiromycins. In *Antibiotics I: Mechanisms of Action*, pp. 211-245. Edited by D. Gottlieb & P. D. Shaw. Berlin, Heidelberg & New York: Springer-Verlag.
- VAN TASSEL, R. L. & WILKINS, T. D. (1978). Isolation of auxotrophs of *B. fragilis*. *Canadian Journal of Microbiology* **24**, 1619-1621.