Pyrimidine Dimer Excision Repair of DNA in *Bacteroides fragilis* Wild-type and Mitomycin C-sensitive/UV-sensitive Mutants

By VALERIE R. ABRATT,¹ GEORGE L. LINDSAY² AND DAVID R. WOODS^{1*}

¹CSIR Microbial Genetics Research Unit, Department of Microbiology and ²Department of Biochemistry, University of Cape Town, Rondebosch 7700, South Africa

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An enzyme preparation purified from *Micrococcus luteus* was shown to be specific for UV-induced pyrimidine dimers and was suitable for the detection of DNA excision repair systems. The wild-type *Bacteroides fragilis* Bf-2 strain and a mitomycin C-sensitive mutant (MTC25) had constitutive dimer excision systems which functioned efficiently under anaerobic and aerobic conditions. A UV-sensitive mutant (UVS9) had markedly reduced levels of the constitutive dimer excision systems under anaerobic and aerobic conditions. Since liquid holding recovery under aerobic conditions was inhibited by chloramphenicol whereas the final level of excision repair in *B. fragilis* Bf-2 was not affected, it is concluded that pyrimidine dimer removal is not the process responsible for increased physiological aerobic liquid holding recovery.

INTRODUCTION

The repair of DNA damage in *Bacteroides fragilis* is interesting in that survival after far-UV irradiation (254 nm) is affected by O₂ (Jones *et al.*, 1980; Jones & Woods, 1981; 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*). The physiological responses of *B. fragilis* to UV damage have been investigated (Jones *et al.*, 1980; Jones & Woods, 1981; Slade *et al.*, 1981; Schumann *et al.*, 1982), but the molecular mechanisms for repair of UV-irradiated DNA in this organism are unknown. We recently reported the isolation and physiological characterization of mitomycin C-sensitive mutants in *B. fragilis* (Abratt *et al.*, 1985). One mutant (MTC25) was markedly sensitive to mitomycin C but had the same UV sensitivity as the wild-type; another mutant (UVS9) was sensitive to UV radiation but was only moderately sensitive to mitomycin C. We have investigated the excision repair capabilities of these mutants and the wild-type strain using the dimer-specific *Micrococcus luteus* UV endonuclease enzyme to detect the presence of pyrimidine dimers.

METHODS

Bacteria. The *B. fragilis* wild-type strain (Bf-2), and mitomycin C-sensitive (MTC25) and UV-sensitive (UVS9) mutants of this strain, were utilized; they have been described previously (Abratt *et al.*, 1985).

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

UV irradiation. Methods for irradiating cells under aerobic and anaerobic conditions were as described previously (Jones & Woods, 1981).

Purification of UV endonuclease from *M. luteus*. This was done by a modification of the methods of Grafstrom *et al.* (1982) and Hays *et al.* (1985). The crude *M. luteus* extract (fraction A of Grafstrom *et al.*, 1982) was loaded onto an Affi-Gel Blue (Biorad) column, bed volume 50 ml, equilibrated with 0.3 M-NaCl in buffer containing 20 mM-Tris/HCl, pH 8.0, 1 mM-EDTA, 1 mM-2-mercaptoethanol and 10% (v/v) glycerol. The column was washed

stepwise with this buffer, first containing 0.3 M-NaCl, then 1.2 M-NaCl; the enzyme activity was then eluted with 3 M-NaCl. The NaCl was removed by dialysis and the partially purified endonuclease precipitated with 70% (w/v) ammonium sulphate. Storage was at -20°C in 50 mM-HEPES/NaOH, pH 7.6, containing 100 mg bovine serum albumin ml^{-1} , 1 mM-dithiothreitol, 0.19% (v/v) Triton X-100 and 50% (v/v) glycerol.

DNA excision repair. Overnight broth cultures of the *B. fragilis* strains were diluted 10-fold into 10 ml prewarmed broth and grown to mid-exponential phase (A_{600} 0.3–0.4). The cells were harvested anaerobically by centrifugation and resuspended in 10 ml anaerobic or aerobic one-quarter-strength Ringer's solution. The cell suspensions were exposed to a UV dose of 15 J m^{-2} (Jones & Woods, 1981). Samples were removed either immediately after irradiation or after post-irradiation incubation at 30°C . The cells were incubated at 30°C as previous physiological experiments on liquid holding recovery were done at 30°C (Jones & Woods, 1981). In experiments to determine the effect of chloramphenicol on dimer excision, $10\text{ }\mu\text{g}$ chloramphenicol ml^{-1} was added to the cells 15 min before irradiation and to the holding buffer after irradiation. Cells from 1 ml samples were harvested by centrifugation, resuspended in 0.5 ml cold lysis buffer (50 mM-Tris/HCl, pH 8.0, 10 mM-Na-EDTA, 500 mM-NaCl), and lysed at 4°C for 10–15 min by the addition of SDS to a final concentration of 1% (w/v). The DNA was extracted with phenol and precipitated with 99% (v/v) ethanol during overnight storage at -20°C . The precipitate was pelleted by centrifugation and resuspended in $100\text{ }\mu\text{l}$ endonuclease buffer (Campbell & Yasbin, 1984). Repair of UV damage was assayed as described by Campbell & Yasbin (1984) (Fig. 1). The UV endonuclease was diluted 1:100 and $2\text{ }\mu\text{l}$ was added to $20\text{ }\mu\text{l}$ DNA. After 1 h incubation at 37°C the entire sample was electrophoresed on a 0.5% alkaline agarose gel (Maniatis *et al.*, 1982). The ethidium bromide stained gel was photographed using Polaroid negative film. Quantification was done by scanning the negative with a Beckman DU 8 spectrophotometer (Wood, 1985). The amount of high M_r DNA present after the various treatments was related to undigested control samples included in each gel by peak triangulation. High M_r DNA is defined as DNA which is no longer sensitive to incision by *M. luteus* UV endonuclease.

RESULTS AND DISCUSSION

Preparation of the M. luteus dimer specific endonuclease

Although *B. fragilis* has been shown to incorporate exogenous radiolabelled thymidine into DNA (Schumann *et al.*, 1984), the level of incorporation is insufficient to allow excision repair studies involving radiolabelled thymine dimers at low UV doses. An alternative approach (Campbell & Yasbin, 1984) using the pyrimidine dimer specific *M. luteus* endonuclease (Riazzudin & Grossman, 1977) was made to detect the presence of pyrimidine dimers in the UV-irradiated DNA. This enzyme makes a single strand cut adjacent to any pyrimidine dimers present in the DNA. If the dimers have been removed by a repair mechanism, the DNA will not be nicked. The enzyme prepared as described was specific for irradiated pBR325 DNA when assayed semi-quantitatively (Hays *et al.*, 1985). A $1\text{ }\mu\text{l}$ sample of a 1:100 dilution of the enzyme (about $0.1\text{ }\mu\text{g}$ protein; Bradford, 1976) completely relaxed 340 ng supercoiled pBR325 DNA irradiated with a dose of 150 J m^{-2} . The enzyme specifically nicked irradiated *B. fragilis* chromosomal DNA; this was detected on the gel as a broad smear of small DNA fragments which sometimes masked the plasmid(s) contained in Bf-2 cells (Fig. 1). The enzyme preparation had no effect on unirradiated *B. fragilis* DNA (Fig. 1) and the chromosomal DNA appeared as a zone of high M_r DNA.

Excision repair of thymine dimers

Irradiation of the cells at 15 J m^{-2} was chosen for these experiments as this is a convenient low dose falling at the end of the shoulder region of the *B. fragilis* wild-type survival curve. Lower doses ($5\text{--}15\text{ J m}^{-2}$) gave similar but less easily visualized results on the gels.

The wild-type *B. fragilis* strain was able to remove pyrimidine dimers during holding in buffer under both anaerobic and aerobic conditions (Fig. 1A, Table 1). Immediately after irradiation the chromosomal DNA was nicked by the *M. luteus* dimer specific endonuclease. After 1 h holding in buffer under aerobic and anaerobic conditions, the DNA was repaired and appeared as a band of high M_r DNA. Densitometer tracings of the gels clearly reflected the gel pictures (Fig. 2). The DNA profiles for nicked and repaired DNA could be easily distinguished and the amount of repair estimated as a percentage of the undigested control of each sample (Table 1). The mitomycin C-sensitive (MTC25) mutant strain also showed dimer removal within 1 h under aerobic and anaerobic conditions (Figs 1B and 2B, Table 1). In comparison with the wild-type,

Fig. 1. Dimer excision by *B. fragilis* Bf-2, MTC25 and UVS9 strains under anaerobic conditions. The presence or absence of dimers in the irradiated DNA was assayed using the dimer specific *M. luteus* UV endonuclease. The enzyme was diluted 1:100 and 2 μ l was added to 20 μ l *B. fragilis* DNA. After incubation at 37 °C for 1 h the entire sample plus 5 μ l alkaline tracking dye was electrophoresed on a 0.5% alkaline agarose gel. A, wild-type; B, MTC25; C, UVS9; D, wild-type unirradiated control. HM_r, high M_r chromosomal DNA; P, plasmid DNA.

Key:

Lane ...	A, B and C						D			
	1	2	3	4	5	6	1	2	3	4
UV (15 J m ⁻²)	+	+	+	+	+	+	-	-	-	-
*HT	0	1	2	0	1	2	0	0	2	2
Endonuclease	-	-	-	+	+	+	-	+	-	+

* HT, Post-irradiation holding time (h).

Table 1. Effect of chloramphenicol on the maximum levels of dimer excision in *B. fragilis*

Chloramphenicol (Cm) (10 μ g ml⁻¹) was added 15 min before irradiation and during holding in buffer under aerobic and anaerobic conditions. The cells were incubated until no further repair took place and the final percentage of high M_r DNA present was determined by triangulation of the peak occurring within 5 mm from the end of the well (Fig. 2). The amount of high M_r DNA in the digested sample after the various treatments was expressed as a percentage of its undigested control. The range of results obtained in four experiments is given.

Strain	High M _r DNA (%)			
	Anaerobic		Aerobic	
	- Cm	+ Cm	- Cm	+ Cm
Wild-type	81-100	85-100	84-98	91-94
MTC25	52-69	46-73	94-100	90-100
UVS9	16-30	18-31	20-48	33-43

the MTC25 mutant showed slightly lower levels of dimer removal under anaerobic conditions. The UV-sensitive (UVS9) mutant showed very much reduced dimer removal under both aerobic and anaerobic conditions (Figs 1C and 2C, Table 1).

The effect of chloramphenicol on the excision of dimers was determined by the addition of 10 μ g chloramphenicol ml⁻¹ 15 min before irradiation and during holding in buffer under anaerobic and aerobic conditions. Schumann *et al.* (1984) showed that this concentration of

Fig. 2. Densitometer tracing of alkaline agarose gels shown in Fig. 1. A, wild-type; B, MTC25; C, UVS9. The ethidium bromide stained gels were photographed using Polaroid negative film and scanned with a Beckman DU8 spectrophotometer. ---, 0 h post-irradiation holding time (lane 4, Fig. 1.); —, 2 h post-irradiation holding time (lane 6, Fig. 1). HM_r , high M_r chromosomal DNA; W, gel well.

chloramphenicol inhibits protein synthesis in this strain. In the presence of chloramphenicol the same maximum level of total repair was ultimately obtained as in the absence of chloramphenicol (Table 1) but it took longer. The rate of repair was approximately halved by chloramphenicol. The rate of repair was also affected by the physiological state of the cells. Cells grown under sub-optimal conditions before irradiation showed slower rates of repair. The addition of chloramphenicol to the holding buffer immediately after irradiation did not affect the rate or final level of repair.

Since dimer excision was not abolished by chloramphenicol it is concluded that there is a constitutive dimer excision system in *B. fragilis*. The decrease in the rate of dimer excision in cells pre-treated with chloramphenicol could be due to either the effect of chloramphenicol on the physiological state of the cells, or to the inhibition of synthesis of another induced dimer excision system which enhanced the rate of repair.

Jones *et al.* (1981) reported that liquid holding recovery occurred in *B. fragilis* Bf-2 only under aerobic conditions and that this was completely inhibited by $10\ \mu\text{g}$ chloramphenicol ml^{-1} added to the holding buffer immediately after irradiation (Jones, 1979). The results reported here show that dimer excision was not inhibited under these experimental conditions. Removal of pyrimidine dimers from irradiated *B. fragilis* DNA was therefore not the process responsible for increased physiological recovery during aerobic liquid holding. This observation was supported by the fact that dimer excision was found to occur under anaerobic conditions where no physiological liquid holding recovery was observed (Jones *et al.*, 1981). Furthermore, the UV-sensitive mutant of *B. fragilis* showed very low levels of dimer excision under aerobic conditions (20–40%), while maintaining the same level of physiological liquid holding recovery as the wild-type (Abratt *et al.*, 1985). This result differs from that found in *Escherichia coli* K12 *recA* mutants where dimer excision is essential for liquid holding recovery (Ganesan & Smith, 1969). The mitomycin C-sensitive mutant showed slightly reduced levels of dimer excision compared with the wild-type under anaerobic conditions (50–70%) in spite of having wild-type resistance to UV. This suggests that dimer removal is not the only repair system functional in *B. fragilis*. The UV-sensitive mutant was not totally deficient in repair. It retained a low level of excision repair and had a residual shoulder (indicating repair) in the UV survival curve at low UV doses (Abratt *et al.*, 1985). The low level of repair in the UVS9 mutant could be interpreted as either evidence for a second repair system or, alternatively, the mutant has a leaky mutation and excision repair is only reduced. However the chloramphenicol experiments indicate that *B. fragilis* possesses at least two UV repair systems; a constitutive dimer excision mechanism, and a further, as yet unidentified, cellular response.

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