RESEARCH LETTER

An AraC/XylS family transcriptional regulator homologue from *Bacteroides fragilis* is associated with cell survival following DNA damage

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Abstract

A putative transcriptional regulator of the AraC/XylS family was identified in a genomic genebank of *Bacteroides fragilis* Bf-1, which partially relieved the sensitivity of *Escherichia coli* DNA repair mutants to the DNA-damaging agents, metronidazole and mitomycin C. A homologue of this gene with the same phenotype was identified as BF638R3281 in *B. fragilis* 638R. Transcription of BF638R3281 was constitutive with respect to exposure to sublethal doses of metronidazole. BF638R3281 was interrupted by single cross-over gene-specific insertion mutation, and the gene disruption was confirmed by PCR and DNA-sequencing analysis. The mutant grew more slowly than the wild type, and the mutation rendered *B. fragilis* more sensitive to metronidazole and mitomycin C. This indicates that the BF638R3281 gene product plays a role in the survival of *B. fragilis* following DNA damage by these agents.

Introduction

Bacteroides species are Gram-negative obligate anaerobes residing in the gastrointestinal tract of mammals, and accounting for approximately one-third of the colonic microbiota (Salyers, 1984). *Bacteroides fragilis* is a medically important opportunistic pathogen, causing more than 80% of anaerobic infections including abscess formation and septicaemia. One of the drugs of choice in treating *B. fragilis* infections is metronidazole, and the level of resistance is currently $< 5\%$ worldwide (Falagas & Siakavellas, 2000). However, there are indications that this is increasing, and a small number of clinical isolates are resistant to metronidazole and other therapeutic agents simultaneously (Turner *et al.*, 1995).

Metronidazole enters cells as an inactive prodrug and is activated intracellularly via anaerobic reduction to form the reactive intermediate, which is a DNA-damaging agent (Edwards, 1977). The mechanisms of resistance to metronidazole described thus far in *Bacteroides* include active drug efflux (Pumbwe *et al.*, 2006), the presence of *nim* genes that code for drug-inactivating nitroreductases (Löfmark *et al.*, 2005), as well as alteration of electron flux by modulation of pyruvate breakdown (Narikawa *et al.*, 1991; Diniz *et al.*, 2004; Gal & Brazier, 2004).

Studies have shown that impairment of DNA repair mechanisms caused increased sensitivity of bacteria to metronidazole, whereas overproduction of the *recA* gene from a plasmid caused increased resistance to the drug (Chang *et al.*, 1997). *Escherichia coli recA* and nucleotide excision repair mutants are more sensitive to metronidazole than the wild-type isogenic strain (Jackson *et al.*, 1984; Yeung *et al.*, 1984), as are *recA* mutants of *Bacteroides thetaiotaomicron*, *Helicobacter pylori* and *Mycobacterium bovis* (Thompson & Blaser, 1995; Cooper *et al.*, 1997; Sander *et al.*, 2001). The elucidation of the DNA repair response of *Bacteroides* to metronidazole exposure could provide valuable information regarding the potential alternative mechanisms of resistance or susceptibility of the bacteria to metronidazole.

In the present study, a twofold approach was used to identify candidate genes involved in the cellular response of *B. fragilis* to metronidazole and mitomycin C (MTC) damage. A genomic *B. fragilis* genebank was screened in a suitable drug-sensitive DNA repair mutant *E. coli* host for a candidate gene conferring resistance. The gene of interest was then specifically mutated in *B. fragilis*, by insertional inactivation, to determine its functional role in the cell response to DNA damage. Here, the isolation and characterization of a putative transcriptional regulator that increased the resistance of *E. coli* DNA repair mutants to metronidazole and MTC is reported, and it is shown that this regulator was also required for wild-type cell responses to these DNA-damaging agents in *B. fragilis*.

Materials and methods

Bacterial strains and plasmids

The plasmids and strains used in this study are described in Table 1. Genbank analysis and primer extension experiments were performed with DNA and RNA derived from *B. fragilis* Bf-1 (Mossie *et al.*, 1979). All subsequent experiments were performed with *B. fragilis* 638R (Privitera *et al.*, 1979), because *B. fragilis* Bf-1 cannot be manipulated genetically. *Bacteroides fragilis* strains were grown at 37 °C in supplemented brain heart infusion (BHIS) medium (Holdeman & Moore, 1972) in an anaerobic chamber (Model 1024, Forma Scientific Inc., Marietta, OH) containing an atmosphere of oxygen-free N_2, CO_2 and H_2 (85 : 10 : 5 by volume). *Escherichia coli* strains were grown aerobically at 37 °C on Luria–Bertani (LB) broth or agar (1.5% w/v) medium (Sambrook *et al.*, 1989) with the addition of ampicillin (100 μg mL^{-1}) where applicable.

Isolation of a plasmid conferring resistance to DNA-damaging agents

Plasmid pAN2 harbouring 4.5 kb of *B. fragilis* Bf-1 chromosomal DNA was isolated from a gene bank (Southern *et al.*, 1986) by screening on Luria agar, containing ampicillin and MTC (range 0.6–1 μg mL^{-1}). This plasmid, or control plasmid pMT104, was transformed into the relevant *E. coli* DNA repair mutant strains and analysed for increased resistance to DNA-damaging agents.

Plasmid constructions

Localization of the functional region of pAN2 was determined by subcloning and deletion analysis. Deletion plasmids pANBB, pANC and pANH (Fig. 1) were constructed by subcloning the 2.5-kb HindIII–BglII fragment, the 1.4-kb HindIII–PvuII fragment and the 1.9-kb HindIII–BamHI fragment from pAN2, respectively, into pEcoR251. Plasmids pRecQ and pE2Reg were constructed by cloning the amplified 2300 and 900-bp fragments from pAN2 into pEcoR251, using primer pairs 1 and 2, respectively (Table 2).

Metronidazole and MTC resistance assays in *E. coli* repair mutants

The agar dilution method was used for drug resistance assays. For aerobic growth, stationary-phase cultures of *E. coli* strains AB1157, AB1886, AB1885 and AB1884,

Table 1. Bacterial strains and plasmids

Strains	Relevant characteristics	Sources or references
B. fragilis Bf-*1*	Metronidazole and MTC susceptible	Mossie *et al*. (1979)
B. fragilis 638R	Metronidazole and MTC susceptible	Privitera *et al*. (1979)
B. fragilis 638R *reg*	Mutant created by insertional inactivation of BF638R3281.	This study
E. coli JM109	*recA1*, Δ*(lac-proAB)[F′,traD36,proAB,lacIZΔM15]*	Setlow *et al*. (1963)
E. coli AB1157	*uvr*$^+$ ATCC29055	Bachmann (1987)
E. coli AB1886	*uvrA6*, derived from AB1157	Howard-Flanders *et al*. (1966)
E. coli AB1885	*uvrB5*, derived from AB1157	Howard-Flanders *et al*. (1966)
E. coli AB1884	*uvrC34*, derived from AB1157	Howard-Flanders *et al*. (1966)
E. coli S17-1	*recA* derivative of *E. coli* 294 (F$^-$ *thi pro hsdR*) carrying a modified derivative of IncPα plasmid pRP4 (Ap^s Tc^s Km^s) integrated in the chromosome, Tp^r	Simon *et al*. (1983)
Plasmids		
pEcoR251	*E. coli* suicide vector,	Zabeau & Stanley (1982)
pMT104	pEcoR251 with a noncoding insert	Wehnert *et al*. (1990)
pAN2	pEcoR251 with 4.5-kb *B. fragilis* genomic fragment	This work
pGERM	*Bacteroides* insertion vector containing *ermG* and *oriT*$_{RK2}$ that is mobilized by RP4 transfer functions in *E. coli* S17-1 chromosome; (Em^r Rep) Ap^r	Cheng *et al*. (2000)
pGAR	pGERM-derivative with BF638R3281 internal fragment	This work

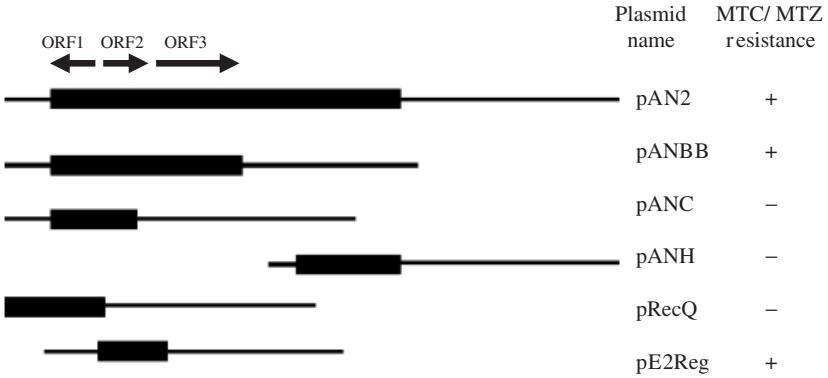

Fig. 1. Genomic organization of pAN2 and deletion plasmids. MTC and metronidazole (MTZ) resistance phenotypes in both *Escherichia coli* AB1886 (*uvrA*) and AB1885 (*uvrB*) are indicated: +, increased resistance as compared with *E. coli* transformed with control plasmid pMT104; –, same level of sensitivity as with pMT104.

Table 2. Oligonucleotide primers

Pair	Name	Sequence (5′–3′)	Purpose	Origin
	PER	CY5GTC GTA CAA TTC ATC TGC	Primer extension	This study
1	Re1	CCT TAG CTG AAT AGT CCG	PCR of full length ORF1	This study
	Re2	CCT ATT GCA ATT GGC AGC		
2	Re3	GGT TGT GGA AGA TCT CTT CC	PCR of full length ORF2	This study
	Re4	CAA GAT TAC GGT TGC AGC		This study
2	AI1	ACA AAA CCC AGG AAG AAA CTC C	To obtain internal fragment from 638RBF3281	This study
	AI2	GCA GCG TAA AAA GAC TGA CGG		This study
3	Re3	As above	To confirm insertional mutation of 638RBF3281	This study
	M13R	GTT TTC CCA GTC ACG AC		Yanisch-Perron *et al*. (1985)
4	Re4	As above	To confirm insertional mutation of 638RBF3281	This study
	M13F	CGC CAG GGT TTT CCC AGT CAC GAC		Yanisch-Perron *et al*. (1985)

containing pAN2 or pMT104, were diluted, and 10^3 cells were plated onto LB–ampicillin agar plates containing either MTC (range 0–2.4 μg mL^{-1}) or metronidazole (range 0–1000 μg mL^{-1}). For anaerobic growth, the same *E. coli* cultures were plated on prereduced yeast–tryptone (YT) agar, containing the antibiotics, and also supplemented with 0.5% glucose and 0.2% sodium nitrate. The minimum inhibitory concentration (MIC) was determined after a 24-h incubation under aerobic or anaerobic conditions.

DNA sequencing and computer analysis

Sequencing reactions were performed using the DYEnamic ET Dye terminator Cycle sequencing Kit for MegaBACE (Molecular Dynamics), based on dideoxynucleotide chain termination chemistry (Sanger *et al*., 1977). The nucleotide sequences were analysed using the MEGABACE 500 SEQUENCE ANALYSER v2.4 software and the software program DNAMAN, version 4.13 (Lynnon BioSoft). Sequence similarity searches were performed using the NCBI BLAST program (Altschul *et al*., 1997).

RNA extraction and analysis

Total RNA was extracted as described previously (Aiba *et al*., 1981). Primer extension analysis was performed to identify the transcriptional start of ORF2. RNA (70 μg) was annealed with Cy5-labelled primer PER (Table 1) at 42 °C overnight. The annealed mixture was subjected to primer extension with 20 U of AMV Reverse Transcriptase (Promega) at 42 °C for 2 h. The primer extension products were sequenced using the ALFexpress Automated DNA Sequencer (Pharmacia Biotech) and analysed together with the sequence products using the same primer PER. For transcriptional analysis of BF638R3281, total RNA was extracted from mid-logarithmic phase cultures of *B. fragilis* 638R exposed to a sublethal dose (1 μg mL^{-1}) of metronidazole. Equal amounts of RNA (4 μL each, containing 10 μg of RNA per spot) were spotted onto nylon membranes and UV cross-linked (Hoefer UVC Cross-linker, Amersham Biosciences). The DNA probe used was an internal fragment of BF638R3281 obtained by PCR using primer pair 2 (Table 2) and digoxigenin-labelled using Digoxigenin (Roche Diagnostics). Hybridization was performed overnight at 50 °C using EasyHyb hybridization buffer and chemiluminescence detected using CSPD (Roche Diagnostics).

Construction and characterization of a *B. fragilis* BF638R3281 mutant

An internal fragment of BF638R3281 was amplified by PCR from *B. fragilis* 638R, using primer pair 2 (Table 2). The

Table 3. Susceptibility of *Escherichia coli* strains, transformed with pAN2, to metronidazole and MTC

E. coli strain	Relevant repair characteristics	Mitomycin C MIC* ($\mu g\,mL^{-1}$)	Metronidazole MIC* ($\mu g\,mL^{-1}$)	
			O_2†	AnO_2†
AB1886 (pAN2)	*uvrA*$^-$	0.7	400	50
AB1886 (pMT104)	*uvrA*$^-$	0.5	300	35
AB1885 (pAN2)	*uvrB*$^-$	1.1	950	250
AB1885 (pMT104)	*uvrB*$^-$	0.7	600	220
AB1884 (pAN2)	*uvrC*$^-$	0.7	300	ND
AB1884 (pMT104)	*uvrC*$^-$	0.7	300	ND

A representative set of data is shown from 10 experiments.

*The MIC was determined on LB agar plates under aerobic conditions.

†The MICs were determined under aerobic conditions (O_2) or anaerobic conditions in prereduced media (AnO_2).

ND, not determined.

fragment was cloned into suicide vector pGERM (Cheng *et al.*, 2000) to create plasmid pGAR, which was transformed into *E. coli* S17-1 and transferred via aerobic mating into *B. fragilis* 638R as described previously (Hooper *et al.*, 1999). Putative mutants were selected anaerobically on BHIS agar containing gentamycin ($200\,\mu g\,mL^{-1}$) and erythromycin ($10\,\mu g\,mL^{-1}$). The mutation locus was confirmed by PCR using primer pairs 3 and 4 (Table 2) to obtain fragments of DNA flanking the plasmid insertion in BF638R3281. The identity of the PCR fragments obtained was verified by sequencing. The mutant was named *B. fragilis* 638R *reg*.

Analysis of growth of *B. fragilis* strains 638R and *B. fragilis* 638R *reg* mutant

The growth of the wild-type and mutant strains in BHIS broth was monitored. The $OD_{600\,nm}$ of the cultures was measured every 2 h for a period of 24 h (Spectrawave S1000, Biochrom). Four independent experiments were performed for each strain.

Sensitivity assays with DNA-damaging agents

Metronidazole ($5\,\mu g\,mL^{-1}$) was added to mid-logarithmic cultures of the *B. fragilis* 638R and *B. fragilis* 638R *reg* mutant. Aliquots were removed from cultures at times from 0 to 40 min. Viable cell numbers were determined under anaerobic conditions by diluting aliquots in anaerobic water, plating on BHIS agar and enumeration of colonies after two days of growth at 37 °C. Survival after 40 min of exposure was expressed relative to survival at 0 min of exposure.

For MTC assays, overnight cultures were centrifuged, and cells were resuspended in prereduced Ringers solution to obtain an $OD_{600\,nm}$ of 0.8. MTC was added to a final concentration of $1\,\mu g\,mL^{-1}$. Aliquots were removed from cultures at times from 0 to 30 min. Dilutions of aliquots were made and plated on BHIS agar to obtain viable cell numbers. For both damaging agents, three independent experiments were performed and the mean percentage survival was calculated (SE in parentheses).

Results and discussion

Identification of a gene involved in metronidazole and MTC resistance

A *B. fragilis* Bf-1 gene library was screened for genes conferring increased metronidazole and MTC resistance in *E. coli uvr* repair mutants. *Escherichia coli* AB1886 *uvrA* transformants with increased MTC resistance relative to the parent strain were isolated on plates containing $0.6\,\mu g\,mL^{-1}$ MTC. These were all found to harbour the same plasmid, carrying a 4.5-kb DNA fragment from the *B. fragilis* chromosome. The recombinant plasmid was designated pAN2 (Fig. 1).

The presence of pAN2 also increased the survival of the *E. coli uvrA* mutant following metronidazole treatment. Similarly, the *uvrB* mutant containing pAN2 survived better than the control after metronidazole and MTC exposure. pAN2 did not affect the *E. coli uvrC* mutant survival following either treatment (Table 3). The improved survival conferred by pAN2 on *E. coli uvrA* and *uvrB* mutants after metronidazole exposure was also present under anaerobic conditions. The fact that the increased survival of metronidazole-treated cells was seen under both aerobic and anaerobic conditions suggested that the cloned gene product might be linked to cell survival following DNA damage caused by the activated metronidazole nitro radicals formed under anaerobic conditions, as well as the reactive oxygen species (ROS) formed during oxidation of metronidazole in the presence of O_2 (Edwards, 1977). This might also indicate that the *B. fragilis* ORF2 is involved in a general stress tolerance mechanism in the heterologous *E. coli*.

Deletion analysis of the 4.5-kb insert showed that only the plasmids pANBB and pE2Reg (Fig. 1) yielded increased resistance to the *E. coli uvrA* and *uvrB* mutants. This

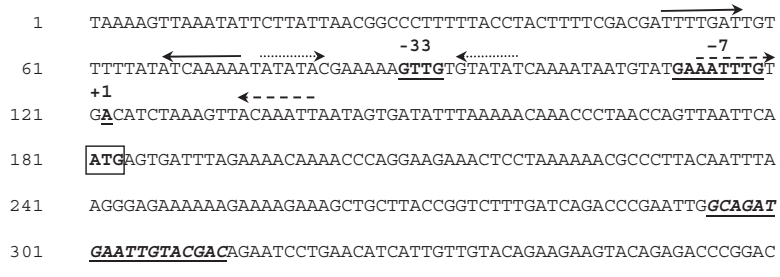

Fig. 2. Identification of the *Bacteroides fragilis reg* transcriptional start site by primer extension analysis. Nucleotide sequence of the putative promoter region of *reg*: the ATG start codon (box) is at position 181, the transcription start site is at (+1) and the putative −7 and −33 motifs are in bold and underlined. The three sets of inverted repeats are shown by matching arrows. The reverse primer used is shown in bold italics.

suggested that the gene involved in the resistance phenotype was found within the common region on these fragments. The DNA insert in plasmid pAN2 that conferred resistance to MTC and metronidazole was fully sequenced in both directions. This region contained an incomplete ORF1, truncated at the C-terminus, and two complete ORFs (ORF2 and ORF3) (Fig. 1). Disruption of ORF2 by deletion analysis caused loss of activity, suggesting the involvement of the ORF2 gene product in resistance. This was confirmed by the fact that the PCR-cloned full-length ORF2 alone caused the improved survival phenotype in *E. coli uvrA* and *uvrB* mutants (data not shown). This gene was designated *reg*.

Sequence analysis and primer extension of *reg*

The transcription start point of *reg* was determined using primer extension analysis. A single primer extension product identified the transcriptional start site to be an adenine 59 bp upstream of the putative ATG start codon of *reg* (Fig. 2). The promoter region revealed the presence of sequences similar to the *B. fragilis* promoter consensus sequences (Bayley *et al.*, 2000) −7 (TGAAATTTG) and −33 (GTTG). Three sets of inverted repeats were identified in the promoter region. One set was found upstream of the −33 promoter sequence, while the others were located near the −33 and −7 promoter regions, which may involve a regulatory role. The *reg* gene encoded a putative protein of 154 amino acids with a deduced molecular weight of 18.1 kDa and sequence similarity at the amino acid level to members of the AraC/XylS family of transcriptional regulators. AraC/XylS regulators typically contain a highly conserved region of 100 amino acid residues that constitutes the DNA-binding domain (Gallegos *et al.*, 1997; Rhee *et al.*, 1998), and the *B. fragilis* Reg contained most of the amino acids of this consensus sequence. The homologues of *reg* in other *B. fragilis* strains are BF3248 in *B. fragilis* NCTC 9343 (Accession number NC_003228) and BF638R3281 in *B. fragilis* 638R (www.sanger.ac.uk), and all three genes are identical in nucleotide sequence and gene arrangement on the chromosome.

The identification of a regulator from this family with potential involvement in DNA repair is significant because a number of these transcriptional regulators have been involved in bacterial virulence (Frota *et al.*, 2004), multidrug resistance systems and in response to alkylating agents (Tanaka *et al.*, 1997). The best-characterized members of this family are the *E. coli* MarA and Rob proteins, both of which have been implicated in multidrug resistance (Gallegos *et al.*, 1997; Kwon *et al.*, 2000). The DNA-binding regulatory domain of these two proteins contains seven α-helices that have been shown to form two helix–turn–helix (HTH) motifs involved in binding to adjacent grooves of the DNA (Bustos & Schleif, 1993). The deduced amino acid sequence of the *B. fragilis* Reg putative protein similarly contained eight α-helices: one outside the conserved domain and seven within the DNA-binding domain, with two putative HTH motifs.

Mutation of the BF3248 homologue in *B. fragilis* 638R

In order to investigate the involvement of the putative regulator in cell survival following DNA damage in *B. fragilis*, a mutation in BF638R3281 (the BF3248 homologue in strain 638R) was achieved by gene-specific insertional inactivation. The *B. fragilis* Bf-1 strain could not be used for this purpose because it cannot be genetically manipulated. The site of insertion was confirmed by PCR analysis of the insertion junction using M13 primers (annealing to the inserted plasmid) in combination with *B. fragilis*-specific primers flanking the mutated gene. The PCR products obtained were sequenced, and this confirmed the insertion of plasmid pGAR into the target gene. Sequence data also revealed that the internal gene fragment, cloned into the suicide vector to facilitate homologous recombination, was now duplicated and flanked the inserted plasmid. The mutant was named *B. fragilis* 638R *reg*.

Growth analysis

The growth of *B. fragilis* 638R and *B. fragilis* 638R *reg* in BHIS broth (Fig. 3) showed that the mutant grew

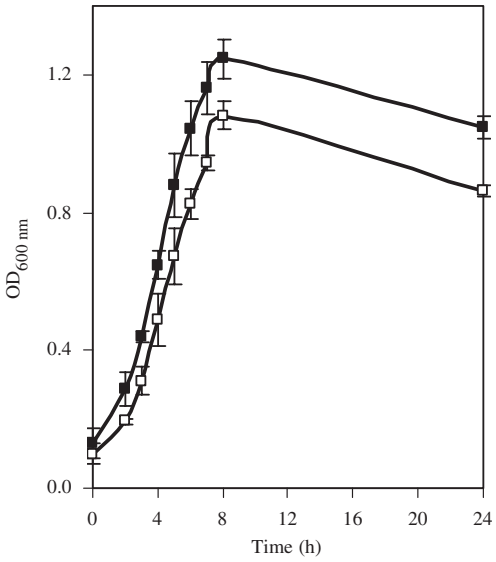

Fig. 3. Analysis of growth of *Bacteroides fragilis* 638R and *B. fragilis* 638R *reg*. The growth of the wild-type *B. fragilis* 638R and *B. fragilis* 638R *reg* mutant strains in BHIS broth was monitored using the $OD_{600\,nm}$ of the cultures. Four independent experiments were performed for each strain. ■, *Bacteroides fragilis* 638R; □, *B. fragilis* 638R *reg*.

more slowly than the parent strain, as measured by $OD_{600\,nm}$. This was most noticeable at the later growth stages, and after 24 h the parent and mutant showed $OD_{600\,nm}$ values of 1.048 (±0.031) and 0.863 (±0.014), respectively. This suggested that the Reg protein is required throughout growth, but might become more important as the age of the culture increases. If Reg is a global regulator, a number of essential cell functions could be affected by its absence. However, it is interesting to note that a slow growth phenotype has been described in certain clinically isolated metronidazole-resistant *B. fragilis* isolates (Gal & Brazier, 2004). It was, therefore, important to determine whether the *reg* gene was itself regulated at the transcription level by exposure to sublethal concentrations of metronidazole, and whether the *B. fragilis* 638R *reg* mutant was more or less sensitive to metronidazole that the parent strain.

Regulation of *reg* transcription by exposure to metronidazole

Transcription of the *reg* gene in *B. fragilis* 638R was analysed during normal growth and after exposure to a sublethal dose of metronidazole (1 μg mL^{-1}). There was no change in the level of transcription of this gene upon exposure to metronidazole relative to the untreated control (data not shown). This indicated that the putative regulator was expressed constitutively under both these conditions.

Sensitivity to metronidazole and MTC

The *B. fragilis* 638R *reg* mutant was more sensitive than the parent strain to both metronidazole and MTC, suggesting that the gene product is involved in protecting the cells against these DNA-damaging agents. This confirmed the results obtained when the cloned gene was introduced into the *E. coli* repair mutants. After 40 min of exposure of mid logarithmic phase cultures to metronidazole, 91.38 (±18.5)% of *B. fragilis* 638R parent cells remained viable, compared with only 8.5 (±4.2)% of the *reg* mutant strain. A similar pattern was observed on exposure to MTC, where only 0.009 (±0.063)% of the mutant cells survived exposure to MTC, compared with 7.62 (±2.75)% of the *B. fragilis* 638R parent strain. These results suggest that *reg* may perhaps be involved in modulating cell responses that repair single-stranded breaks (caused by metronidazole) and cross-linked DNA (caused by MTC).

DNA single- and double-strand breaks are repaired in Gram-negative bacteria via the RecFOR and RecBCD pathways, respectively, through the helicase and exonuclease functions of the proteins involved. Mutations in these pathways cause increased sensitivity of *E. coli* to DNA-damaging agents such as MTC (Keller *et al.*, 2001). Sensitivity to MTC in Gram-negative bacteria is caused by mutations in *recA*, defective nucleotide excision repair, as well as defective *recBCD* and *recFOR* repair pathways (Keller *et al.*, 2001; Zuniga-Castillo *et al.*, 2004). No information has been published to date regarding the sensitivity of *B. fragilis recBCD* mutants or *recFOR* mutants to metronidazole. This report, therefore, describes a novel role for a putative AraC/XylS transcriptional regulator with regard to the damage induced by the DNA-damaging agents, metronidazole and MTC. Further studies will elucidate which genes are regulated by the Reg protein, and whether they are damage specific or linked to general cell stress responses. The work will provide valuable information concerning the ways in which *B. fragilis* manages the DNA damage caused by metronidazole and MTC exposure.

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