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Porphyromonas gingivalis mutY is involved in the repair of oxidative stress-induced DNA mispairing

A.G. Robles[1], K. Reid[2], F. Roy[1] and H.M. Fletcher[1]

1 Division of Microbiology and Molecular Genetics, School of Medicine, Loma Linda University, Loma Linda, CA, USA
2 Department of Biology, Chemistry and Allied Health Science, College of Naturaland Applied Sciences, Northern Caribbean University, Mandeville, Jamaica, West Indies

Correspondence: Antonette G. Robles, Division of Microbiology and Molecular Genetics, School of Medicine, Loma Linda University, Loma Linda, CA 92350, USA Tel.: +1 909 558 4472; fax: +1 909 558 4035; E-mail: agrobles@llu.edu

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SUMMARY

The ability for DNA mismatch repair, after oxidative stress-induced DNA damage, is critical for the persistence of *Porphyromonas gingivalis* in the inflammatory environment of the periodontal pocket. Our previous report demonstrated that, in contrast to other organisms, the repair of oxidative stress-induced DNA damage involving 8-oxo-7,8-dihydroguanine (8-oxoG) may occur by a yet-to-be described mechanism in *P. gingivalis*. 8-oxoG does not block DNA replication; rather, it mispairs with adenine, which can be repaired by the MutY glycosylase. To determine the function of the *P. gingivalis* MutY homologue in DNA repair, it was insertionally inactivated using the *ermF-ermAM* antibiotic cassette and used to create a *mutY*-deficient mutant (FLL147) by allelic exchange mutagenesis. FLL147 had an increased rate of spontaneous mutation and was more sensitive to hydrogen peroxide compared with the wild-type W83 strain. DNA oligomers containing a site-specific 8-oxoG:A mispair was repaired similarly in both the *P. gingivalis mutY*-defective mutant and wild-type strains. The *P. gingivalis mutY* homologue was shown to complement the *mutY* mutation in *Escherichia coli*. In a gel mobility shift assay, the purified recombinant MutY is able to bind an oligo containing an 8-oxoG:A mispair. Taken together, MutY may play the expected role in oxidative stress resistance in *P. gingivalis.* However, there may exist other redundant mechanism(s) for the removal of 8-oxoG:A mismatch in this organism.

INTRODUCTION

Porphyromonas gingivalis is a gram-negative, black-pigmented, obligate anaerobic rod that is the major pathogen associated with periodontitis, a chronic inflammatory disease (Lamont & Jenkinson, 1998). In the inflammatory microenvironment of the periodontal pocket, reactive oxygen species (ROS) generated from polymorphonuclear leukocyte and macrophage activities represent a major source of oxidative stress (Chapple, 1996). The ROS can damage proteins, lipids, RNA and DNA (Miller & Britigan, 1997; Canakci *et al.*, 2005). More specifically, ROS can negatively affect the base component of a nucleotide triphosphate or a nucleotide already incorporated within DNA (Miller & Britigan, 1997). The most extensively studied DNA lesion is 8-oxoguanine (8-oxoG), which is the major product of DNA exposure to oxidative stress (Sekiguchi & Tsuzuki, 2002). Lethal DNA

mutations can result from this base damage because 8-oxoG does not block DNA replication; rather, it mispairs with adenine, resulting in GC to TA transversions (Grollman & Moriya, 1993; Tchou & Grollman, 1993). Hence, it is imperative that *P. gingivalis* develop an appropriate mechanism to overcome ROS-induced DNA damage.

In general, most oxidative DNA damage, whether it is base or sugar damage or the formation of abasic sites, is repaired by base excision repair (BER) (Krokan *et al.*, 1997). DNA lesions generated as a result of ROS damage are usually not bulky DNA lesions and are excellent substrates for the process of BER (Gros *et al.*, 2002). DNA *N*-glycosylases are the effectors of BER and are more specific in lesion recognition and repair than the process of nucleotide excision repair (Krokan *et al.*, 1997). Most glycosylases cleave the *N*-glycosidic bond between the abnormal base and the deoxyribose resulting in the formation of an abasic site, which is further processed by abasic site endonuclease, DNA polymerase, DNA ligase and additional enzymes (Lu *et al.*, 2001).

BER is well studied in *Escherichia coli* and involves MutM, MutY and MutT (the GO system), which protect the cell from the mutagenic effects of 8-oxoG (Michaels & Miller, 1992). MutM removes 8-oxoG paired with cytosine (Chung *et al.*, 1991). If replication occurs without the removal of 8-oxoG, MutY, another glycosylase, removes adenine mispaired with 8-oxoG (Au *et al.*, 1989). The MutM then has a second chance of removing 8-oxoG paired with cytosine. Hence, MutM and MutY work together to prevent GC to TA transversions associated with 8-oxoG. MutT hydrolyses 8-oxo-dGTP to 8-oxo-dGMP, depleting the nucleotide pool of 8-oxo-dGTP (Maki & Sekiguchi, 1992). MutS, part of methyl directed mismatch repair, can also repair 8-oxoG:A mispairs to 8-oxoG:C (Tchou & Grollman, 1993; Wyrzykowski & Volkert, 2003). Nei and Nth can both serve as back-up proteins for MutM (Hazra *et al.*, 2000; Morland *et al.*, 2002; Takao *et al.*, 2002).

In contrast, the repair mechanisms for DNA damage resulting from ROS are unclear in *P. gingivalis*. The *P. gingivalis* does not contain MutM or Nei homologues. We have previously reported that the removal of 8-oxoG may not occur in *P. gingivalis* by BER, as is observed in other organisms (Johnson *et al.*, 2004). A novel mechanism may be involved in the repair of the 8-oxoG lesion. UvrB, known to be important in nucleotide excision repair, is also not involved in its removal (Henry *et al.*, 2008). Instead, it is likely that other unique protein(s) may be involved in that repair process. Unlike MutM and Nei, *P. gingivalis* does contain homologues to MutY, MutT and MutS. MutY from *P. gingivalis* is 51% similar to *E. coli* MutY. Because MutY is known to repair 8-oxoG:A in *E. coli*, its function in *P. gingivalis* was further investigated. The results indicate that MutY may play a role in *P. gingivalis* similar to that observed in other bacteria. However, it is also likely that a redundant mechanism may exist for the repair of 8-oxoG:A mispair in *P. gingivalis*.

METHODS

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this experiment are listed in Table 1. *Porphyromonas gingivalis* strains were grown in brain–heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with hemin (5 μg ml^{-1}), vitamin K (0.5 μg ml^{-1}) and cysteine (1%). The *E. coli* strains were grown in Luria–Bertani (LB) broth (Difco Laboratories). Unless otherwise stated, all cultures were incubated at 37°C. The *P. gingivalis* strains were maintained in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) in 10% H_2, 10% CO_2, 80% N_2. Antibiotics were used at the following concentrations: erythromycin, 10 μg ml^{-1} in *P. gingivalis* and 300 μg ml^{-1} in *E. coli*; carbenicillin, 100 μg ml^{-1} and rifampicin 1 μg ml^{-1} (spontaneous mutagenicity assay) or 100 μg ml^{-1} (rifampicin-resistance mutation assay).

Bioinformatics of MutY

The amino acid sequence of *P. gingivalis* W83 MutY was obtained from http://www.oralgen.lanl.gov. A sequence alignment comparing *P. gingivalis* MutY (strain W83) with *E. coli* MutY (strain K-12) was performed using CLUSTALW (v.1.81) (Chenna *et al.*, 2003).

Generation of *mutY* mutant

The 2.2-kb fragment containing the *mutY* gene and flanking regions was amplified from *P. gingivalis* chromosomal DNA (W83) using primers P1 and P2, (Table 2) by polymerase chain reaction (PCR). The

Table 1 Plasmids and bacterial strains used in this study

	Phenotype/description	Source
Plasmids		
pCR-XL-TOPO 3.5	Ap^r, Km^r	Invitrogen
pFLL145	*mutY* gene cloned into pCR-XL-TOPO 3.5	This study
pFLL146	*ermF-ermAM* cloned into *Sma*I site of *mutY* in pFLL145	This study
pVA2198	Sp^r, *ermF-ermAM*	(Fletcher *et al.*, 1995)
pET11a	Ap^r	Novagen
pGEV1	Ap^r, G protein N-terminal tag, C-terminal His tag	Addgene, (Huth *et al.*, 1997)
pCR-XL-TOPO-PG *mutY*	*P. gingivalis mutY* cloned into pCR-XL-TOPO	This study
pET11a-PG *mutY*	*P. gingivalis mutY* cloned into pET11a	This study
pGEV1-PG *mutY*	*P. gingivalis mutY* cloned into pGEV1	This study
Bacterial strains		
W83	Wild type *P. gingivalis*	(Abaibou *et al.*, 2001)
FLL147	*mutY* defective mutant	This study
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ (*lacZYA-argF*) *U169 recA1 endA1 hsdR17* ($r_K^-m_K^+$) *phoA supE44 thi-1 gyrA96 relA1*	Invitrogen
PR8	*E. coli* wild-type	A-Lien Lu-Chang
PR70(DE3)	*E. coli mutY* mutant	A-Lien Lu-Chang
BL21(DE3)	*E. coli* B F^- *dcm ompT hsdS* ($r_B^-m_B^-$) *gal*λ (DE3)	Stratagene

mutY gene was TOPO TA cloned into pCR-XL-TOPO, 3.5-kb (Invitrogen, Carlsbad, CA) and subsequently transformed into DH5α *E. coli* competent cells (Invitrogen). One construct with the *mutY* gene in the desired orientation was designated pFLL145 and chosen for further manipulation. The *ermF-ermAM* antibiotic cassette was PCR amplified from pVA2198 using *Pfu* turbo polymerase (Stratagene, Cedar Creek, TX) and was ligated into the *Sma*I site of *mutY* in pFLL145. The resulting plasmid, pFLL146 was used as a donor in electroporation of *P. gingivalis* W83 for homologous recombination as previously described (Abaibou *et al.*, 2001), creating a *mutY* mutant; one colony designated as FLL147 was chosen for further study.

DNA isolation and analysis

Extraction of *P. gingivalis* chromosomal DNA and plasmid DNA was performed as previously described

Table 2 Primers and oligonucleotides used for this study

	Description	Sequence
Primer		
P1	*mutY* forward	5′-CGGTTACGAATATGCCCAGA-3′
P2	*mutY* reverse	5′-AATTTGGTGCGTGCCTTATC-3′
P3	*erm* forward	5′-TATTAGGCCTATAGCTTCCGCTATT-3′
P4	*erm* reverse	5′-AATTAGGCCTTAGTAACGTGTAAGTTT-3′
P7	*vimA* reverse	5′-TACCTGTTTTTGCTGACCGG-3′
P8	*vimA* forward	5′-ATGCCCATCCCTCTATACCTG-3′
Oligo		
O1	Glycosylase assay	5′-GAACTAGTGOATCCCCCGGGCTGC-3′3′-CTTGATCACATAGGGGGCCCGACG-5′
O2	Glycosylase assay	5′-GGCTATCGTGGCUGGCCACGACGG-3′3′-CCGATAGCACCGACCGGTGCTGCC-5′
O3	Glycosylase assay	5′-CCGTCGTGGCCOGCCACGATAGCC-3′3′-GGCAGCACCGGACGGTGCTATCGG-5′
O4	EMSA	5′-CCGAGGAATTAGCCTTCTGC-3′3′-GGCTCCTTAAOCGGAAGACG-5′

O1, O2 and O3 were each labeled with ^{32}P at the 5′ end of the strand containing the lesion of interest.
O4 was biotinylated at both 5′ ends.
O, 8-oxoG.

(Vanterpool *et al.*, 2004). For large-scale preparation, plasmids were purified using a Qiagen plasmid maxi kit (Quagen, Santa Clarita, CA).

Confirmation of inactivation of *mutY* via PCR and RT-PCR

For PCR, using chromosomal DNA from three different *mutY*-defective erythromycin-resistant colonies and primers (Table 2) specific to *mutY* (P1 and P2) or to *ermF-ermAM* (P3 and P4), PCR amplification was performed using High-Fidelity PCR Master enzyme mix (Roche, Indianapolis, IN). Plasmids pFLL145 and pVA2198 were used as positive controls. The PCR amplified DNA was analyzed via 1% agarose gel electrophoresis.

For reverse transcription (RT) -PCR, total RNA was extracted from *P. gingivalis* W83 and *P. gingivalis* FLL147 grown to mid-log phase [optical density at 600 nm (OD_{600}) of 0.7] using the RiboPure kit (Ambion, Austin, TX). The RT-PCR was performed using primers P1 and P2 and Superscript One-Step RT-PCR mix (Invitrogen). Primers P7 and P8 were used in the positive controls. Reverse transcription and PCR amplification were performed with a Perkin-Elmer Cetus DNA thermal Cycler (Perkin Elmer Corporation, Norwalk, CT). The final products were analyzed using 1% agarose gel electrophoresis.

Spontaneous mutagenicity assay

Mutagenesis, as measured by development of rifampicin resistance, was determined for W83 and FLL147. Overnight cultures were used to inoculate BHI broth and allowed to grow to mid log phase (OD_{600} 0.6). One hundred microliters of the undiluted samples was plated on BHI plates containing 1 μg ml^{-1} rifampicin. The plates were then incubated under anaerobic conditions at 37°C for 4–5 days. Cell titer was determined using standard viable plate count methods with samples plated on BHI plates. Serial dilutions were performed (Barker, 1998) using standard procedures. Mutation rates were calculated as the number of rifampicin-resistant colonies to 10^{10} cells plated (Lu-Chang, 2006). The experiment was performed six separate times using independent cultures.

Preparation of crude bacterial extracts

Porphyromonas gingivalis strains W83 and FLL147 were grown in BHI broth at 37°C to an OD_{600} of 0.6. Each strain was treated with hydrogen peroxide (0.25 mM) for 15 min. The *E. coli* was grown in a similar manner under aerobic conditions without the addition of hydrogen peroxide. The cell pellets were collected by centrifugation at 15,000 ***g*** for 1 h at 4°C. Each cell pellet was treated with complete protease inhibitors (Roche), resuspended in lysis buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA), subjected to eight freeze–thaw cycles, and then sonicated twice, for 30 s each time. Cell debris was removed by centrifugation at 15,000 ***g*** for 20 min at 4°C. The protein concentration of the supernatant was determined using Pierce's BCA Protein Assay Kit (Thermo-Scientific, Rockford, IL).

Escherichia coli strains PR8, PR70(DE3), PR70(DE3)/pET11a and PR70(DE3)/pET11a-PG MutY were grown in 500 ml LB broth at 37°C, with appropriate antibiotics when needed. At an OD_{600} of 0.7, the cells were induced with 0.1 mM isopropylthiogalactoside (IPTG) for 2.5 h. The cells were then processed according to Lu *et al.* (1983).

Oligonucleotide labeling and annealing procedures

Oligonucleotides used in this study were purchased from Trevigen (Gaithersburg, MD) and IDT Technologies (Integrated DNA Technologies, San Diego, CA). Both the 5′-end labeling with ^{32}P and the annealing procedures were performed as previously described (Johnson *et al.*, 2004); ^{32}P was purchased from MP Biomedicals, Inc. (Irvine, CA).

Glycosylase assay

^{32}P-labeled and annealed oligonucleotides (1 pmol) were incubated with *P. gingivalis* or *E. coli* cell extracts (2 μg total protein), or *E. coli* MutY or uracil-*N*-glycosylase (amount used as per product instructions) in 1× enzyme buffer (supplied with the *E. coli* MutY and uracil-*N*-glycosylase; Trevigen) or *E. coli* MutY glycosylase buffer (Lu-Chang, 2006). The reactions were incubated at 37°C for 5 min. Then, 1 μl 1 M NaOH was added and each reaction was incubated at

90°C for 30 min. Then, 5 μl formamide dye was added and the samples were incubated at 90°C for 2 min.

Gel electrophoresis and analysis of cleavage of glycosylase assay

Reaction samples were loaded onto 20% denaturing polyacrylamide gels (7 M urea) and run for 90 min at 500 V. The resulting bands corresponding to the cleavage products and uncleaved substrate were visualized using a Molecular Dynamics Phosphor Imager (Amersham Biosciences, Piscataway, NJ) and IMAGEQUANT 5.0 software (GE Healthcare, Piscataway, NJ).

Hydrogen peroxide sensitivity assay

Strains W83 and FLL147 of *P. gingivalis* were grown to early log phase in BHI broth without cysteine with appropriate antibiotics, as needed. Each culture was split in two and one half of the culture was treated with hydrogen peroxide at a final concentration of 0.25 mM whereas the other half was not treated with hydrogen peroxide and served as a control. All cultures were further incubated for a total of 28 h. At specific time intervals, a sample of each culture was taken and the titer was determined for each strain under each condition using standard viable plate count methods to determine % survival of each strain. Three independent experiments were conducted.

Complementation: rifampicin-resistance mutation assay

The rifampicin-resistance mutation assay was performed as previously described (Lu-Chang, 2006). The *E. coli* strains PR8 and *mutY* mutant strain PR70(DE3), which expresses a truncated MutY lacking the C-terminal domain were provided by Dr. A-Lien Lu-Chang (Li & Lu, 2003). PR70(DE3) displays a mutator phenotype. PR8 served as a control. The *P. gingivalis mutY* was cloned into expression vector pET11a using standard cloning procedures (pET11a-PG *mutY*), sequenced, and then transformed into PR70(DE3). PR70(DE3) was also transformed with pET11a and used as a control. Cells were grown to an OD_{600} of approximately 0.5 with appropriate antibiotics, as needed, and the pellet was used to inoculate fresh LB media the following day. Cells were grown to an OD_{600} of 0.7 and then induced with 0.1 mM IPTG for 2.5 h. Cells were plated on LB agar plates containing 100 μg ml^{-1} rifampicin and grown for 24 h at 37°C. The cell titer was determined using standard viable plate count methods. The mutation frequency was calculated as previously described (Lu-Chang, 2006). The experiment was performed three separate times in duplicate.

Cloning, expression, and purification of *mutY*

Porphyromonas gingivalis mutY was amplified by PCR, cut with *Nhe*I and *Xho*I, and then ligated into the pGEV1 vector cut with the same restriction enzymes using standard molecular biology techniques. The pGEV1 vector was obtained from Addgene (http://www.addgene.org). Cloning of *mutY* into pGEV1 results in an N-terminal fusion with streptococcal protein G and a C-terminal fusion with His_6. The resulting construct, pGEV1-PG *mutY*, was sequenced and then transformed into BL21(DE3) for expression. The pGEV1 transformed into BL21(DE3) was used as a negative control. Cells were grown to an OD_{600} of approximately 0.5 and then induced with IPTG at a final concentration of 0.2 mM for 4 h at room temperature. Cells were harvested via centrifugation and then frozen at −20°C. GB1-PG MutY-His_6 was purified using a Ni-NTA column purchased from Qiagen (Valencia, CA). Protein was dialysed against buffer containing 0.025 M HEPES, pH 7.6, 0.1 mM EDTA, 2 mM dithiothreitol and 100 mM KCl to remove imidazole.

Gel mobility shift assay

The gel mobility shift assay was performed with purified protein from BL21(DE3)/pGEV1 (negative control) and BL21(DE3)/pGEV1-PG MutY and cell extract from *E. coli* strain PR8 (positive control) using Pierce's (Thermo Fisher Scientific, Rockford, IL) LightShift Chemiluminescent EMSA kit with some modifications as per Dr A-Lien Lu-Chang (Li & Lu, 2003). The biotin-labelled 20-mer DNA substrate containing 8-oxoG mispaired with A was purchased from Eurogentec North America, Inc. (San Diego). The following amounts were used in the assay: 20 fmol biotin-labeled 20-mer DNA, 4 pmol unlabelled oligo, 2 μg protein.

RESULTS

Bioinformatic analysis suggests that *E. coli* MutY and *P. gingivalis* MutY have conserved structural features

The MutY from *P. gingivalis* has 51% similarity with MutY from *E. coli*, so it was predicted that the two proteins would contain important conserved functional regions. Using CLUSTALW (v.1.81), a sequence alignment was performed using both proteins (Fig. 1). The catalytic residues in the active site are indicated with black arrows (Glu37 and Asp138 in *E. coli* MutY) and are present in both proteins. Val45 and Gln182, recently shown to be important in adenine specificity in *E. coli* MutY, are also conserved in *P. gingivalis* MutY. In addition, the *P. gingivalis* MutY contains the following domains, which are also found in *E. coli* MutY: helix-hairpin-helix, iron-sulfur cluster loop, and endonuclease III. The iron-sulfur domain in *P. gingivalis mutY* has the four conserved Cys residues present in *E. coli* MutY. Taken together, these observations suggest that the MutY proteins from *P. gingivalis* and *E. coli* may have conserved structural features.

Inactivation of *P. gingivalis mutY* in W83

To further clarify the role of the *P. gingivalis mutY* gene, an isogenic mutant defective in that gene was constructed using allelic exchange mutagenesis. Three isogenic mutants were randomly chosen for further analysis. All colonies plated on *Brucella* blood agar were black pigmented and exhibited β-hemolysis. These isogenic mutants also displayed similar growth rates and gingipain activities compared with the wild-type strain (data not shown).

PCR and RT-PCR analysis of *P. gingivalis* W83 and *mutY* defective mutants

Confirmation of the inactivated *mutY* gene was performed using PCR (data not shown). If the *mutY* gene (2.2 kb) was interrupted by the *ermF-ermAM* cassette (2.1 kb), a 4.3-kb fragment was expected to be amplified in contrast to a 2.2-kb fragment using

Figure 1 Sequence alignment comparing *Porphyromonas gingivalis* MutY (PG) with *Escherichia coli* MutY (EC). The catalytic residues in the active site are indicated by black triangles (Glu37 and Asp138). Identical residues are marked with an asterisk (*) whereas highly conserved residues are marked with a colon (:). The blue line above the sequence (PG) or below the sequence (EC) indicates the helix-hairpin-helix domain; the green line indicates the endonuclease domain; the red line indicates the iron-sulfur cluster domain. The sequence alignment indicates that the two proteins may have similar functions.

chromosomal DNA as the template and primers P1 and P2 specific for *mutY* (Table 2). A 4.3-kb fragment was indeed amplified from the three different erythromycin-resistant *mutY* colonies. Further, using primers P3 and P4 specific for the *ermF-ermAM* cassette, the expected 2.1-kb fragment was amplified from the three *P. gingivalis mutY* deletion mutants. One *mutY* deletion mutant colony designated *P. gingivalis* FLL147 was randomly chosen for further study. To further validate the inactivation of the *mutY* gene in *P. gingivalis* FLL147, the absence of the *mutY* transcript was verified using RT-PCR and primers P1 and P2 specific for *mutY* (data not shown). In contrast to W83, there was no amplified fragment corresponding to the *mutY* gene in FLL147. The *vimA* gene was used as a positive internal control and using *vimA* specific primers, it was amplified in both W83 and FLL147. The *vimA* gene is a putative acyl-CoA *N*-acyltransferase or is predicted to have sortase-like activity. Inactivation of the *mutY* gene did not have any polar effects on downstream genes (data not shown).

FLL147 has an increased rate of spontaneous mutation vs. W83

The effect of *mutY* on the spontaneous mutation rate in *P. gingivalis* was evaluated by selection of rifampicin-resistant mutants. After plating the cells on BHI plates containing 1 μg ml^{-1} rifampicin, W83 contained two rifampicin-resistant colonies per 10^{10} cells plated (± 1) whereas FLL147 had 93 resistant colonies per 10^{10} cells plated (± 8). The experiment was performed six times with six independent cultures. Hence, the *mutY* defective mutant (FLL147) had a 47-fold increase in spontaneous resistance to rifampicin when compared with W83.

FLL147 is more sensitive to hydrogen peroxide than W83

The sensitivity of FLL147 to 0.25 mM hydrogen peroxide was compared with wild-type W83 by determining the percentage of viable cells after treating bacterial cells with 0.25 mM hydrogen peroxide. As seen in Fig. 2, FLL147 was more sensitive to hydrogen peroxide than the wild-type W83 strain. Taken together, the results indicate that *mutY* is important in protecting the cell from oxidative stress.

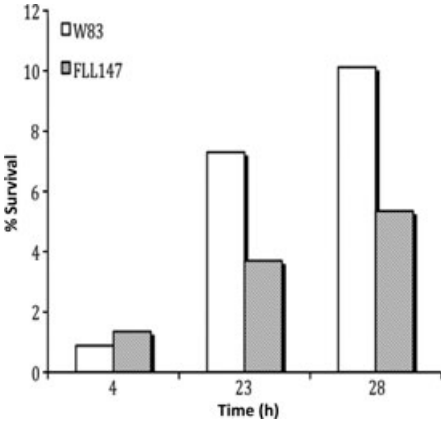

Figure 2 Per cent survival of W83 versus FLL147 after treatment with 0.25 mM hydrogen peroxide. The experiment was performed three times. The survival rate of W83 was found to be twice that of FLL147. The graph is representative of typical results obtained.

DNA oligomers containing a site-specific 8-oxoG:A mispair is repaired similarly using cell extracts from FLL147 and W83

Porphyromonas gingivalis W83 and the isogenic mutant *P. gingivalis* FLL147 were assessed to determine whether inactivation of *mutY* altered the enzymatic removal of A from 8-oxoG:A mispair. Bacterial extracts from the *P. gingivalis* isogenic strains grown in the presence of hydrogen peroxide were used in glycosylase assays with [γ-^{32}P]ATP-5′-end-labeled DNA oligomers (24-mer) containing a site-specific 8-oxoG:A mispair (Oligo O3, Table 2). If the A is enzymatically removed from the 8-oxoG:A mispair by BER, a fragment the size of a 12-mer should be observed. As shown in Fig. 3A, lane 1, the *E. coli* MutY enzyme generated a 12-mer fragment. Using the same oligo substrate and cell extracts from FLL147 and W83, a cleavage band representing a 12-mer was also observed (Fig. 3A, lanes 5 and 6, respectively). The smaller band in lanes 5 and 6 may be the result of nuclease activity. As a control, oligomers (Oligo O2, Table 2) containing U:A mismatches were used to determine the presence of uracil glycosylase activity. As shown in Fig. 3A, lanes 3 and 4, the levels of activity for uracil glycosylase were similar for both W83 and FLL147. The following data suggest that adenine is still removed from an 8-oxoG:A mispair in the *P. gingivalis mutY*-defective mutant.

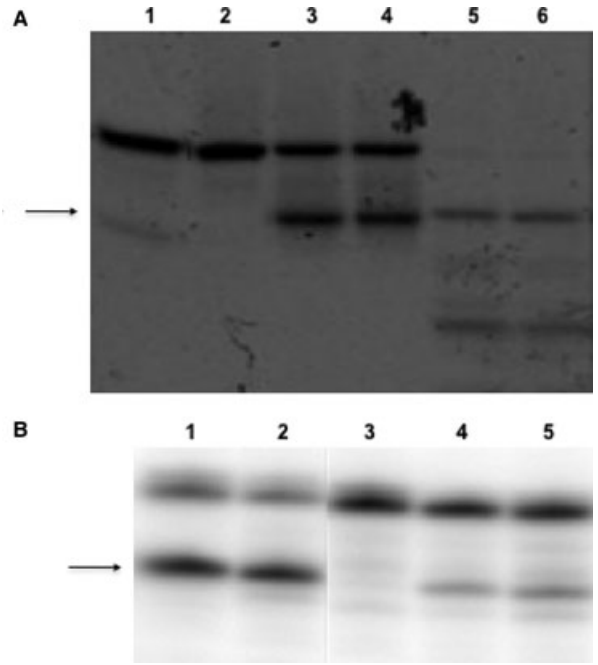

Figure 3 Glycosylase assays (A) Assessing MutY glycosylase activity in W83 versus FLL147 using Oligo O3 (24 mer): lane 1, positive control using purified *Escherichia coli* MutY; lane 2, negative control for MutY glycosylase activity containing no MutY; lane 3, W83 extract assayed for uracil-*N*-glycosylase activity; lane 4, FLL147 extract assayed for uracil-*N*-glycosylase activity; lane 5, FLL147 extract assayed for MutY glycosylase activity; lane 6, W83 extract assayed for MutY glycosylase activity (B) Assessing MutY glycosylase activity to determine if *Porphyromonas gingivalis* MutY is functionally interchangeable with *E. coli* MutY using Oligo O1 (24-mer): lane 1, positive control using purified *E. coli* MutY; lane 2, PR8 cell extract containing a functional *E. coli* MutY; lane 3, PR70(DE3)/pET11a extract lacking a functional MutY; lanes 4 and 5, PR70(DE3)/pET11a-PG *mutY* extract containing a functional *P. gingivalis* MutY. Arrows indicate cleavage product resulting from glycosylase activity. For each glycosylase reaction, the following was used: 1 pmol oligo, 2 μg protein from cell extract or 1 unit purified enzyme.

Porphyromonas gingivalis mutY is able to complement an *E. coli* mutY mutant *in vivo*

Because MutY from *P. gingivalis* has 51% similarity with the *E. coli* MutY, its ability to complement a defect of that gene in *E. coli* was further evaluated. The *E. coli* PR70(DE3) is a *mutY*-defective mutant and therefore fails to correct replication errors, resulting in an increase in rifampicin-resistant cells (Li *et al.*, 2000). Without a functional MutY, an increase in the spontaneous mutation rate at the rifampicin binding site of the RNA polymerase causes the cell to become resistant to rifampicin. The *P. gingivalis mutY* was cloned into the expression vector pET11a and the recombinant plasmid used to transform PR70(DE3). The PR70(DE3) transformed with vector alone was used as a control. In Table 3, the wild-type *E. coli* PR8 strain containing a functional MutY produced very few rifampicin-resistant colonies, as expected. In contrast, PR70(DE3) plated on solid medium in the presence of rifampicin gave rise to spontaneously resistant colonies. When PR70(DE3) was transformed with expression vector pET11a alone, the number of rifampicin-resistant colonies was comparable to PR70(DE3). However, when PR70(DE3) was complemented with *P. gingivalis mutY* (PR70(DE3)/pET11a-PG *mutY*), the number of rifampicin-resistant colonies was restored to levels similar to wild-type, suggesting that *P. gingivalis* MutY is functionally interchangeable with the *E. coli* MutY. To further confirm their functional similarity, cell extracts from the *E. coli* strains were prepared and used in a glycosylase assay (Oligo O1, Table 2). As shown in Fig. 3B, lane 2, PR8 produces the expected 14-mer cleavage band. A similar band was missing in PR70(DE3) transformed with the expression vector pET11a (Fig. 3B, lane 3). However, cell extract from PR70(DE3) transformed with pET11a-PG *mutY* (Fig. 3B, lanes 4 and 5) produces the same cleavage band (14-mer) as PR8, further suggesting that *P. gingivalis* MutY and *E. coli* MutY are functionally equivalent. As the only difference between PR70 *(E. coli* strain without a functional MutY) and PR70/pGEV1-PGmutY is the presence of *P. gingivalis* MutY expressed from pGEV1-PGmutY, it is likely that the glycosylase activity observed in this strain is the result of this protein.

Table 3 Complementation of *Escherichia coli mutY* mutant with *Porphyromonas gingivalis mutY*

Strain	Rifampicin-resistant colonies per 10^8 cells
PR8	4 ± 5
PR70(DE3)	60 ± 8
PR70(DE3)/pET11a	45 ± 17
PR70(DE3)/pET11a-PG *mutY*	2 ± 2

The experiment was performed three separate times in duplicate. The average number of rifampicin-resistant colonies (± SD) per 10^8 cells is reported.

P. gingivalis MutY binds to an oligo containing an 8-oxoG:A mispair

If *P. gingivalis mutY* plays a similar role to *E. coli mutY* in repairing oxidative DNA damage, we next predicted that *P. gingivalis* MutY would be able to bind an oligonucleotide containing an 8-oxoG:A mispair. This would be consistent with bioinformatic results, which demonstrate that *P. gingivlalis* MutY contains the Val45 and Gln182 residues recently shown in *E. coli* MutY to be important for adenine-specific activity (Chang *et al.*, 2009). We tested this hypothesis using a gel mobility shift assay. First, *P. gingivalis mutY* was cloned into expression vector pGEV1 and purified, resulting in *P. gingivalis* MutY being fused with streptococcal protein G at the N terminus and a 6-His tag at the C terminus. As a negative control, the same procedure was used with cells transformed with pGEV1 only. Figure 4, lanes 1–3 shows the functionality of the EMSA kit using the manufacturer's controls and lanes 5 and 6 show that both the *E. coli* MutY and the *P. gingivalis* recombinant MutY proteins can bind the 8-oxoG:A mispair biotin-labelled oligo. To show the specificity of the *P. gingivalis* MutY–DNA interaction, 8-oxoG:A mispair oligo without the biotin label was added in excess. The signal shift observed in lane 6 was prevented by competing with excess unlabeled DNA as seen in lane 7; this indicates the specificity of the interaction. In addition, in a negative control, when protein purified from cells transformed with pGEV1 only were incubated with the labeled 8-oxoG:A oligo, no upward shift was observed (data not shown). Protein–DNA complexes with *E. coli* MutY migrate slightly lower than *P. gingivalis* MutY because *E. coli* MutY has a smaller molecular weight (39 kDa) than *P. gingivalis* MutY (46.8 kDa). Also, the faint band seen at the top of Fig. 4, lane 5, probably represents non-specific binding of protein in the *E. coli* extract containing MutY with the biotin-labeled oligo. Taken together, these results suggest that the *P. gingivalis* MutY can specifically bind to an oligo containing 8-oxoG:A mispair, which would be consistent with its function as an adenine glycosylase.

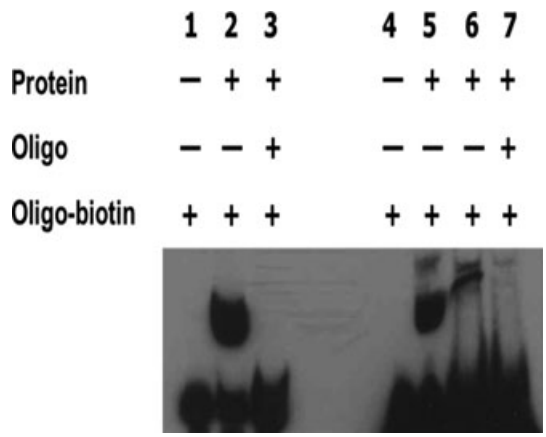

Figure 4 *Porphyromonas gingivalis* MutY binds to an oligo containing an 8-oxoG:A. Lanes 1–3 represent the kit controls using EBNA (Epstein–Barr nuclear antigen) as the protein and EBNA oligo with or without biotin. In lanes 4–7, an oligo containing an 8-oxoG:A mispair with or without biotin was used. Lane 5 contains *Escherichia coli* MutY. Lanes 6 and 7 contain purified *P. gingivalis* MutY. For lanes 4–7, the following was used: 20 fmol oligo-biotin, 4 pmol unlabelled oligo, and 2 μg protein.

DISCUSSION

In this study, we examined the role of the *P. gingivalis mutY* gene in the repair of oxidative stress-induced DNA mispairing. MutY is a significant part of an important system that functions via BER in the repair of DNA mispairing as a consequence of DNA damage induced by ROS (Michaels & Miller, 1992). *Porphyromonas gingivalis* has a single *mutY* gene, which is homologous to the *mutY* genes from other organisms, including *E. coli* (51%) and *Bacteroides fragilis* (45%) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Our previous report has demonstrated that, in contrast to other organisms, the repair of oxidative stress-induced DNA damage involving 8-oxoG may occur by a yet-to-be described mechanism in *P. gingivalis* (Henry *et al.*, 2008). This has raised questions on the role of *P. gingivalis* MutY in the repair process as there is evidence that formation of complexes of *E. coli* MutY with DNA containing an 8-oxo-G mispaired lesion may modulate excision of 8-oxo-G by MutM (Kurthkoti *et al.*, 2010). Perhaps in *P. gingivalis,* MutY is involved in a similar fashion by interacting with another protein equivalent in function to *E. coli* MutM.

Inactivation of the *P. gingivalis mutY* gene resulted in a significant increase in sensitivity to hydrogen peroxide. This correlated with an increase in the spontaneous mutation rate compared with the wild-type strain. These results suggest that DNA mispairing/damage is not repaired as well in the *mutY*-defective

mutant in *P. gingivalis*. These results are consistent with other pathogenic bacteria such as *Helicobacter pylori* (Eutsey *et al.*, 2007) and *Neisseria* sp. (Davidsen *et al.*, 2005). This is in contrast to a MutY-deficient *Mycobacterium smegmatis* strain that has shown no significant increase in spontaneous mutation rate (Kurthkoti *et al.*, 2010). Collectively, these observations would support multiple pathways for the repair of DNA mispairing or damage induced by oxidative stress in *P. gingivalis* because inactivation of MutY results in increased sensitivity to hydrogen peroxide and increased spontaneous mutation rate, but not complete inhibition of bacterial growth. The *E. coli* isogenic mutants defective in the *mutT*, *mutY* or *mutM* genes resulted in an increase in spontaneous mutation frequency, with the *mutT* having the highest mutation frequency and *mutM* having the lowest (Fowler *et al.*, 2003). Whereas *mutM* is missing in *P. gingivalis,* the functional role of *mutY* in this organism is unclear.

The *P. gingivalis* MutY was shown to complement an *E. coli mutY*-defective mutant. The spontaneous mutation rate of *E. coli* PR70(DE3) was returned to wild-type levels when transformed with the *P. gingivalis* MutY. In addition, MutY glycosylase activity was demonstrated in those cells. Taken together, these results suggest that the two proteins have functional similarity and the *P. gingivalis* MutY is functional in a heterologous host. Similar results were obtained when an *E. coli mutY* mutant was complemented with MutY from *H. pylori* (Eutsey *et al.*, 2007), *Neisseria* sp. (Davidsen *et al.*, 2005), *Pseudomonas aeruginosa* (Oliver *et al.*, 2002) and *Deinococcus radiodurans* (Li & Lu, 2001). BER is an important mechanism for repairing oxidative DNA damage (Lu *et al.*, 2001) so it is not surprising that bacteria would possess protein homologues involved in BER. A comparative analysis of DNA repair glycosylases shows that MutY is present in most of the bacterial genomes surveyed (Denver *et al.*, 2003).

Although the *mutY* gene was inactivated in FLL147, there was a similar level of adenine glycosylase activity in the mutant compared with the wild-type W83 strain. This is in contrast to results obtained from *Neisseria* sp., in which whole cell extracts from *mutY* mutants did not exhibit adenine glycosylase activity compared with the wild-type strain (Davidsen *et al.*, 2005). This could suggest that another redundant system might be present in *P. gingivalis* that might be involved in the repair of the 8-oxoG:A mispair. In *E. coli*, MutS can also repair 8-oxoG:A mismatches to 8-oxoG:C as MutY does (Lu *et al.*, 2001). A MutS homologue exists in *P. gingivalis* but its functional significance is still unclear. Interestingly, preliminary microarray analysis indicates that *mutS* is upregulated in *P. gingivalis* FLL92 (non-pigmented *vimA*-defective mutant) compared with wild-type W83 when exposed to hydrogen peroxide (data not shown). This seems to suggest that *P. gingivalis* MutS may play a role in protecting the cell against oxidative stress. *Escherichia coli* MutS is part of the methyl-directed mismatch repair system which has been shown to repair base pairs containing oxidative lesions (Wyrzykowski & Volkert, 2003). Alternatively, the repair system in *P. gingivalis* could be novel and may involve undefined protein(s). The repair of the 8-oxoG lesion in *P. gingivalis* requires a novel complex of proteins (Henry *et al.*, 2008). If there is an interaction between the repair of both the 8-oxoG lesion and 8-oxoG:A mispairing as seen in mycobacteria (Kurthkoti *et al.*, 2010), then we cannot rule out the possible involvement of other novel proteins in this process. This is under further investigation in the laboratory.

Gel mobility shift assays indicate that purified *P. gingivalis* MutY is able to bind to an oligo containing an 8-oxoG:A mispair. This is consistent with results that indicate that *P. gingivalis* MutY is functionally interchangeable with *E. coli* MutY. As seen in bioinformatic analysis, *P. gingivalis* contains two residues recently shown in *E. coli* to be important for DNA substrate binding and specificity for adenine: Val45 and Gln182 (Chang *et al.*, 2009). The *E. coli* MutY has adenine glycosylase activity with A:G, A:C and A:GO mispairs, and weak guanine glycosylase activity with a G:GO mispair (Lu *et al.*, 1995). In *D. radiodurans,* this substrate specificity is conserved (Li & Lu, 2001). The *D. radiodurans* MutY contains Val45 and has a comparable polar amino acid in place of Gln182 (Li & Lu, 2001). MutY from *D. radiodurans* and *P. gingivalis* share significant identity at the C terminus with MutY from *E. coli*. The C terminus of *E. coli* MutY is involved in recognizing specific lesions whereas the N terminus is involved in catalytic activity. The *P. gingivalis* MutY may have similar substrate specificities to *E. coli* MutY in addition to 8-oxoG:A but this remains to be tested.

Taken together, the results presented in this paper indicate that *P. gingivalis* MutY plays an important role in oxidative stress resistance. The results imply that a system involving MutY is a primary mechanism for repair of 8-oxoG:A in addition to another redundant mechanism(s). This could explain the increased susceptibility of the MutY mutant compared with the wild-type to hydrogen peroxide, the increase in spontaneous mutation rate, and the similarity in 8-oxoG:A repair.

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