Repair of oxidative DNA damage in Gram-positive bacteria: the *Lactococcus lactis* Fpg protein

Patrick Duwat,¹ Regina de Oliveira,² S. Dusko Ehrlich¹
and Serge Boiteux²

Author for correspondence: Serge Boiteux. Tel: +33 1 45 59 64 05. Fax: +33 1 46 78 41 20.

¹ Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy en Josas, France

² Groupe 'Réparation des lésions radio et chimioinduites', Centre National de la Recherche Scientifique URA147, Institut Gustave-Roussy, 94800 Villejuif, France

The formamidopyrimidine DNA glycosylase gene (*fpg-L*) of the Gram-positive microaerophilic bacterium *Lactococcus lactis* subsp. *cremoris* ML3 has been cloned, characterized and sequenced. The *fpg-L* gene is composed of 819 bp encoding a protein of 31·3 kDa (Fpg-L). The deduced amino acid sequence of the Fpg-L protein shows 59% similarity and 38% identity with the *Escherichia coli* Fpg protein (Fpg-E). Polyclonal antibodies against Fpg-E react with the Fpg-L protein. The Fpg-L protein was purified to apparent homogeneity from the overproducing *E. coli* strain BH410 hosting plasmid pVE1064, which carries *fpg-L* under the control of the *E. coli* *lac* promoter. In its active form, Fpg-L is a 30 kDa monomeric enzyme with a measured isoelectric point of 9·0. It contains one zinc per molecule and has a zinc finger motif localized at the carboxy-terminal end (Cys-X₂-Cys-X₁₆-Cys-X₂-Cys-X₃-COOH). The Fpg-L protein has two enzyme activities: DNA glycosylase, which excises 2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine and 7,8-dihydro-8-oxoguanine, and DNA nicking at abasic sites. Furthermore, the expression of the *fpg-L* gene in *fpg* and *mutY* mutants of *E. coli* suppresses their spontaneous GC → TA mutator phenotype. The similarity of the activity of the two Fpg proteins and its conservation in evolutionarily distant bacteria may reflect the importance of its role in protecting bacterial DNA against oxidative free radicals.

Keywords: *Lactococcus lactis*, DNA repair, oxidative DNA damage, Fpg protein, *fpg* gene

INTRODUCTION

Reactive oxygen-derived species (ROS) formed in cells either as a byproduct of aerobic metabolism or during oxidative stress have been suggested to play an important role in biological processes such as mutagenesis, carcinogenesis and ageing (Breimer, 1990; Ames *et al.*, 1993; Boiteux, 1993). Amongst ROS, the hydroxyl radical (OH[•]) is highly reactive and produces a complex pattern of DNA modifications (Dizdaroglu, 1991). To counteract the lethal and mutagenic effects of DNA damage induced by ROS, cells have developed DNA repair mechanisms (Sancar & Myles, 1989). Both base excision repair and

nucleotide excision repair are involved in the removal of oxidized bases in DNA (Lin & Sancar, 1989; Czeizot *et al.*, 1991). In *Escherichia coli*, two DNA glycosylases excise oxidized bases, the pyrimidine-specific Nth protein (Breimer & Lindahl, 1985; Dizdaroglu *et al.*, 1993), and the purine-specific Fpg protein. *In vitro*, the *E. coli* Fpg protein (Fpg-E) releases 2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine (Fapy) and 7,8-dihydro-8-oxoguanine (8-OxoG) residues (Chetsanga & Lindahl, 1979; Breimer, 1984; Boiteux *et al.*, 1984, 1992; Tchou *et al.*, 1991; Castaing *et al.*, 1993). Fpg-E also possesses two further activities: DNA incision at apurinic/aprimidinic (AP) sites (Bailly *et al.*, 1989; Boiteux *et al.*, 1990b), and excision of terminal 5'-deoxyribose phosphate (Graves *et al.*, 1992). *In vivo*, the Fpg-E protein repairs lethal and promutagenic oxidative DNA damage (Czeizot *et al.*, 1991; Tudek *et al.*, 1993). Furthermore, *fpg* (*mutM*) mutants of *E. coli* have a spontaneous GC → TA mutator phenotype (Michaels *et al.*, 1992; Castaing *et al.*, 1993) that is greatly enhanced when combined with *mutY* mutations (Michaels *et al.*, 1992). These enzymes, Fpg and MutY,

Abbreviations: AP, apurinic/aprimidinic; Fapy, 2,6-diamino-4-hydroxy-5*N*-methylformamidopyrimidine; Fpg-E protein, Fpg protein of *Escherichia coli*; Fpg-L protein, Fpg protein of *Lactococcus lactis*; FPLC, fast protein liquid chromatography; 8-OxoG, 7,8-dihydro-8-oxoguanine; pBS, pBluescript KS(+); ROS, reactive oxygen-derived species.

The EMBL/GenBank accession number for the nucleotide sequence of *fpg-L* is X74298.

together with MutT form the GO system that prevents mutagenesis by oxidized guanine residues in DNA and in the nucleotide pool (Michaels & Miller, 1992; Grollman & Moriya, 1993).

Lactococcus lactis subsp. *cremoris* is a Gram-positive micro-aerophilic bacterium that has a 2300 kb genome with a G + C content of 37 mol% (LeBourgeois *et al.*, 1992), and it is extensively used in the food industry. The amelioration of the dairy strains needs a molecular approach for solving industrial problems such as the recovery of growth after freezing, and the non-reproducibility of fermentations. Here, we report the molecular cloning and DNA sequencing of the *fpg* gene (*fpg-L*), and the purification of the Fpg protein (Fpg-L) from *L. lactis* ML3. The results suggest that the *fpg-L* and the *fpg-E* genes have issued from a common ancestor. Furthermore, we show that Fpg protein has a highly conserved function in prokaryotic cells.

METHODS

Bacterial strains, plasmids and enzymes. *E. coli* strains used were TG1 and JM105 (Sambrook *et al.*, 1989), BH410 (as

JM105 but *fpg-1::kan^R*) (Boiteux & Huisman, 1989; Tudek *et al.*, 1993), CC104 [*ara* Δ(*gpt-lac*)5 (*F' lacI378 lacZ461 proA⁺B⁺*)] (Cupples & Miller, 1989), PR70 [*micA(mutY)::kan^R*] (Radicella *et al.*, 1988), BH540 (as CC104 but *fpg-1*), BH980 (as CC104 but *mutY*) and BH990 (as CC104 but *fpg-1 mutY*) (this study). *E. coli* strains were grown in LB broth at 37 °C, and genetic manipulations of bacteria were performed as described by Miller (1972). *L. lactis* ML3 strain ML3 (Duwat *et al.*, 1992) was grown on M17 glucose medium (Terzaghi & Sandine, 1975). The pBluescript KS(+) (pBS) plasmid was obtained from Stratagene. Plasmid pVE1001 is a derivative of pBS and contains the *L. lactis recA* gene and, upstream, a truncated ORF that is homologous to that of the *E. coli* Fpg protein (Duwat *et al.*, 1992). The Fpg-E protein was prepared as described previously (Boiteux *et al.*, 1990b). Restriction enzymes, DNA polymerase, DNA ligase, T4 polynucleotide kinase were purchased from Boehringer Mannheim or Appligene, and used as recommended by the manufacturer.

Cloning and sequencing of the *fpg-L* gene. All DNA manipulations were performed according to published methods (Sambrook *et al.*, 1989; Duwat *et al.*, 1992). An *L. lactis* ML3 genomic library was constructed using purified *EcoRI*–*Clal* DNA fragments of 1.5–2 kb, blunted by phage T4 DNA polymerase and cloned into *SmaI*-linearized pBS. *E. coli* TG1 was transformed with the library and ampicillin-resistant transformants were colony hybridized using Hybond-N nylon membrane (Amersham). The probe used was a ³²P-labelled 550 bp *SpeI*–*Clal* DNA fragment of plasmid pVE1001 (see Fig. 1a). For DNA sequencing, different restriction fragments were subcloned from pVE1064. DNA sequences were determined on double-stranded DNA by the dideoxy chain-termination method. The sequence reported was determined on both strands.

Purification of the Fpg-L protein. LB broth (5 l) containing 50 µg ampicillin ml⁻¹ was inoculated with 50 ml of an overnight culture of *E. coli* BH410(pVE1064) plasmid. The cells were grown at 37 °C until OD₆₀₀ 1.0 and supplemented with 0.5 mM IPTG. The IPTG-induced culture was incubated at 37 °C for 20 h. The cells were harvested, washed and stored at –70 °C. The bacteria (13 g) were lysed in buffer A [50 mM HEPES/KOH, pH 7.6, 1 mM Na₂EDTA, 5 mM β-mercaptoethanol and 5% (v/v) glycerol] containing 0.25 M NaCl. The lysate was centrifuged at 30000 r.p.m. for 60 min at 2 °C. The supernatant was the crude extract fraction (fraction 1, 140 ml). Fraction 1 was loaded onto a QMA anion-exchange column (Waters ACCELL, 75 ml bed volume) equilibrated with buffer A containing 0.25 M NaCl. The Fpg-L protein was not retained in these conditions (fraction 2, 300 ml). Fraction 2 was dialysed against buffer A containing 50 mM NaCl, applied to a phospho Ultrogel (PCU) cation-exchange column (IBF–LKB, 20 ml bed volume). The active fractions eluted at 0.25 M NaCl (fraction 3, 87 ml). Fraction 3 was precipitated with ammonium sulfate (500 g l⁻¹), resuspended in 7 ml buffer A without glycerol but containing 1 M NaCl, and loaded on a gel filtration column (IBF–LKB Aca54; 1.6 × 150 cm). The active fractions were pooled (fraction 4, 36 ml). Fraction 4 was supplemented with ammonium sulfate to a final concentration of 0.85 M and loaded onto a Phenyl Sepharose CL-4B column (Pharmacia, 10 ml bed volume). The column was washed with buffer B (50 mM HEPES/KOH, pH 7.6, 1 mM, Na₂EDTA, 5 mM β-mercaptoethanol, 1 M NaCl and 0.85 M ammonium sulfate), and eluted with a linear gradient (0–100% buffer A). The active fractions were pooled (fraction 5, 16 ml). Fraction 5 was dialysed against buffer A containing 50 mM NaCl, and loaded onto an FPLC cationic MonoS HR5/5 column (Pharmacia). The active fractions eluted at 0.3 M NaCl (fraction 6, 9.5 ml).

Fig. 1. Cloning of the *fpg-L* gene. (a) Physical map of the *fpg-recA* locus of *L. lactis* ML3. The arrows indicate the direction of transcription. Positions of the restriction endonuclease sites and the 550 bp probe used to clone the *fpg-L* gene are indicated. (b) Physical map of pVE1064 containing the *fpg-L* gene. The black area corresponds to the *fpg-L* gene, the hatched area to other lactococcal sequences. Other sequences are from the pBS plasmid vector.

Inhibition of Fpg-E and Fpg-L by polyclonal antibodies against Fpg-E protein. Antibodies were prepared as described by Boiteux *et al.* (1990b) and affinity-purified from rabbit antisera using an Fpg-E protein Sepharose column. Five nanograms of purified Fpg-E or Fpg-L protein were incubated with anti-Fpg-E antibodies for 10 min at 20 °C in assay buffer. Then [³H]Fapy-poly(dG-dC) substrate was added and the Fapy DNA glycosylase activity was measured as described.

Assay for the repair of 8-OxoG. A 34-mer oligonucleotide containing a single 8-OxoG residue was used as substrate: 5'-GGCTTCATCGTTATT(8-OxoG)ATGACCTGGTGGA-TACCG-5'. The nucleotide at the 3' end of this modified 34-mer was inverted, yielding the sequence 5'-(N)₃₃-3'-P-3'-N-5' (Castaing *et al.*, 1993). Both ends of this 34-mer were acted on by the T4 polynucleotide kinase and [γ -³²P]ATP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹), Amersham]. 5'-³²P-labelling, assay conditions and PAGE analysis were as described previously (Castaing *et al.*, 1993).

RESULTS

Cloning of the *fpg-L* gene

Sequence analysis of an *L. lactis* DNA fragment containing the *recA* gene revealed, immediately upstream from *recA*, a truncated ORF which would encode a polypeptide having homology with the carboxy-terminal end of the *E. coli* Fpg protein (Boiteux *et al.*, 1987; Duwat *et al.*, 1992). This upstream DNA fragment was used as probe for restriction mapping and cloning of the *fpg-L* gene (Fig. 1a). Considering the size of the *E. coli* *fpg* gene (807 bp) and the location of the *ClaI* restriction site in the lactococcal *recA* sequence, a 1.6 kb *EcoRI*-*ClaI* DNA fragment was expected to contain the entire *fpg-L* gene (Fig. 1a). For cloning, *L. lactis* chromosomal DNA was digested with *EcoRI* and *ClaI* and DNA fragments from 1.5–2.0 kb were recovered, blunted with T4 DNA polymerase and ligated with the *SmaI*-linearized pBS vector. White ampicillin-resistant transformants were selected and colony hybridized. Plasmid DNA was extracted from colonies which gave a strong hybridization signal with the DNA probe used. Fig. 1(b) shows the physical map of pVE1064, which contains the *fpg-L* gene placed under the control of the *E. coli* *lac* promoter.

Nucleotide sequence of the *fpg-L* gene

The 1615 bp DNA fragment cloned in pVE1064 consists of a potential ORF of 819 bp and the 5' region of the lactococcal *recA* gene (Fig. 1). The potential ORF codes for a protein of 273 amino acids with a molecular mass of 31.3 kDa. The putative protein coded by the *fpg-L* gene shows 59% similarity and 38% identity with the Fpg-E protein. The sequence upstream of the 819 bp ORF contains two potential RNA-polymerase-binding sites and a translation initiation site. The DNA sequence between positions 1 and 1158 will appear in the EMBL and GenBank database with the accession number X74298. The sequence from position 1159–1615 was published by Duwat *et al.* (1992).

Sequence comparison with the Fpg-L protein

Database searches revealed four sequences with significant

homology with the Fpg-L protein: the Fpg-E protein, an ORF of *Bacillus firmus* (Ivey, 1990), the amino-terminal portions of ORFs from *Synechocystis* sp. PCC 6803 (Chitnis *et al.*, 1989) and *Synechococcus* sp. PCC 6301 (Rhiel & Bryant, 1992) (Fig. 2). The amino-terminal part was reported for these four proteins and all possess an identical stretch of eight amino acids (PELPEVET) (Fig. 2). No functional role has been attributed to this region. In addition, the carboxy-terminal end of three of these proteins was determined and shows a zinc finger consensus sequence whose organization is conserved (Cys-X₂-Cys-X₁₆-Cys-X₂-Cys) (Fig. 2). In *E. coli*, the four cysteines in the zinc finger motif of the Fpg protein are important, since mutation in any of them results in proteins which are unable to bind DNA and catalyse enzymic reactions (O'Connor *et al.*, 1992; Castaing *et al.*, 1993).

Overproduction and purification of the Fpg-L protein

To overproduce the protein coded by *fpg-L*, plasmid pVE1064 was transformed into *E. coli* BH410 which has a disrupted chromosomal *fpg* gene (Boiteux & Huisman, 1989). Without IPTG-induction of the *lac* promoter, a low level of Fapy DNA glycosylase activity was detected in BH410(pVE1064) crude extracts. Addition of IPTG to the culture derepresses the *lac* promoter and allows the level of Fapy DNA glycosylase activity to increase by more than 200-fold as compared with the uninduced level. These results show that the *fpg-L* gene encodes an enzyme, the Fpg-L protein, endowed with Fapy DNA glycosylase activity.

Twelve milligrams of apparently homogeneous Fpg-L protein were prepared from 13 g IPTG-induced cells (Table 1). The final fraction appeared as a single protein band on SDS-PAGE with a molecular mass of about 30 kDa (Fig. 3). A unique amino-terminal sequence was determined and the first 22 amino acids perfectly match the sequence deduced from the nucleotide sequence, confirming that *fpg-L* is the structural gene of the Fpg protein of *L. lactis*. The Fpg-L protein is basic, with a measured isoelectric point of 9.0. Atomic absorption spectroscopy analysis revealed, as expected, that there is one zinc per Fpg-L protein molecule.

Polyclonal antibodies against the Fpg-E protein recognize Fpg-L protein

Polyclonal antibodies against the Fpg-E protein inhibited the Fapy DNA glycosylase activity of the Fpg-L protein. However, a 10–20-fold excess of antibodies was required to inhibit Fpg-L activity as compared to the amount required to inhibit Fpg-E protein (Fig. 4).

Fpg-L protein nicks DNA at AP sites and excises 8-OxoG

The results reported above suggest a strong conservation between the Fpg-E and Fpg-L proteins. Several lines of evidence indicate that the Fpg-L protein is endowed with an AP nicking activity. Firstly, both the Fapy DNA glycosylase activity and an EDTA-resistant AP nicking

Fig. 2. Comparison of the deduced amino acid sequences of the *fpg* genes from *E. coli* (COLIFPG), *L. lactis* (LACFPG), *Bacillus firmus* (FIRMFPG), *Synechococcus* sp. (SYNFPG) and *Synechocystis* sp. (SYNECFPG). Amino acid positions correspond to the *E. coli* Fpg protein. A star indicates identity. An amino acid is shown in the consensus sequence (consens) if it is identical in at least three sequences. The amino acids in bold are discussed in the text.

duplexes with cytosine, thymine or guanine opposite 8-OxoG (Fig. 5). In contrast, as with Fpg-E, the rate of incision of the duplex with adenine opposite 8-OxoG was much slower (Fig. 5). In all cases, the two fragments generated by Fpg-L were identical to those produced by Fpg-E. These results suggest that the mechanisms of incision at an AP site by Fpg-L and Fpg-E are identical (Bailey *et al.*, 1989). The release of free 8-OxoG, due to DNA glycosylase activity of the Fpg-L protein, was directly confirmed by HPLC analysis of the reaction products (data not shown).

Table 1. Purification of the Fpg-L protein from 13 g IPTG-induced *E. coli* BH410(pVE1064)

Fapy DNA glycosylase and AP site nicking activities were measured using [^3H]Fapy-poly(dG-dC) and partially depurinated [^3H]thymine DNA as substrate, respectively (Boiteux *et al.*, 1984, 1990b). Control IPTG-induced BH410(pBS) crude extract had no detectable Fapy DNA glycosylase activity and 0.6 kU mg $^{-1}$ of AP site nicking activity.

Step	Protein (mg)	Fapy DNA glycosylase activity (kU mg $^{-1}$)	AP site nicking activity (kU mg $^{-1}$)	R*
1	1946	2.1	30.6	15
2	1860	2.3	32.3	14
3	74	39.9	529.1	13
4	28	55.3	835.7	15
5	14	103.9	1600.1	15
6	12	108.2	1687.5	16

* R, AP site nicking activity/Fapy DNA glycosylase activity.

Fig. 3. SDS-PAGE analysis of the Fpg-L protein purification fractions. Lane M, molecular mass markers, phosphorylase *b* (94 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and B-lactalbumin (14 kDa). Lanes 1–6 correspond to the different purification steps described in Table 1 and in Methods. PAGE was performed using 15% (w/v) polyacrylamide gel. The amounts of Fpg-L protein loaded on the gel were: lanes 1 and 2, 50 μg ; lane 3, 10 μg ; lanes 4, 5 and 6, 5 μg . Lane 7, 5 μg purified Fpg-E protein.

Biological function of the Fpg-L protein

To investigate the biological role of the Fpg-L protein, we analysed its capacity to suppress the spontaneous mutator phenotype of *fpg* and *mutY* mutants of *E. coli*. The *E. coli* strains used were derived from CC104, which has a *lacZ* mutation that can only revert through a GC $\rightarrow$ TA transversion event (Cupples & Miller, 1989). Wild-type, *fpg* or *mutY* single mutants, and an *fpg mutY* double mutant were transformed with pVE1064, which contains a functional *fpg-L* gene under the control of the *lac* promoter. Table 2 shows that the presence of the

Fig. 4. Inhibition of Fpg-L and Fpg-E proteins by antibodies against Fpg-E protein. Inhibition of the Fapy DNA glycosylase activity of Fpg-L (○) and Fpg-E (●) proteins by antibodies against Fpg-E protein. Residual activity was expressed as percentage of control activity without antibodies. Up to 2 ml preimmune serum had no detectable inhibitory effect.

Fig. 5. Repair of 8-OxoG by the Fpg-L and Fpg-E proteins. A 34-mer oligonucleotide containing a single 8-OxoG at position 16 was used as a substrate for Fpg-L and Fpg-E. The 8-OxoG-containing strand was ^{32}P -labelled at both ends and annealed with a complementary strand containing T, C, A or G opposite 8-OxoG (*G). The annealing reaction was assessed by neutral PAGE [20% (w/v) polyacrylamide] which indicated that > 95% of the labelled strand was hybridized. Labelled duplexes (40 fmol) were incubated at 37 $^{\circ}\text{C}$ for 5 min with 0.5 ng purified Fpg-L or Fpg-E proteins. The products of the reaction were analysed by PAGE (20% polyacrylamide) with 7 M urea. PPRD1 and PPRD2 indicate the position of the bands generated by chemical cleavage at 8-OxoG by 1 M piperidine.

Fpg-L protein greatly reduces spontaneous mutagenesis in *fpg*, *mutY* and *fpg mutY* strains of *E. coli*. The fact that the Fpg-L protein can substitute for the *E. coli* enzyme

Table 2. Spontaneous GC → TA mutagenesis in *fpg* and *mutY* mutants of *E. coli* expressing the *fpg*-L gene from plasmid pVE1064

Five or more independent overnight cultures of *E. coli* hosting pVE1064 or pBS (the vector from which pVE1064 was derived) were grown in LB broth containing ampicillin. Aliquots were plated on LB agar containing ampicillin (100 µg ml⁻¹) and rifampicin (100 µg ml⁻¹) or on minimal glucose or minimal lactose plates containing ampicillin only (Cupples & Miller, 1989).

Bacterial strain	Frequency of GC → TA transversion*	
	Rif ^R	Lac ⁺
Hosting pBS:		
CC104 (wild-type)	5	2
BH540 (<i>fpg</i>)	19	44
BH980 (<i>mutY</i>)	162	480
BH990 (<i>fpg mutY</i>)	1651	13530
Hosting pVE1064:		
CC104 (wild-type)	4	3
BH540 (<i>fpg</i>)	5	6
BH980 (<i>mutY</i>)	34	86
BH990 (<i>fpg mutY</i>)	84	280

* Values are expressed as Rif^R transvariants per 10⁸ cells and Lac⁺ transvariants per 10⁹ cells.

suggests that these two proteins have similar biological function.

DISCUSSION

The *fpg*-L gene of *L. lactis* is predicted to encode a protein of 273 amino acids with 59 % similarity and 38 % identity with the *E. coli* Fpg protein, Fpg-E. Comparison of physical, catalytic, immunological and biological properties of Fpg-L and Fpg-E shows that these two proteins have highly conserved structure and function. Both Fpg-L and Fpg-E are monomeric proteins which possess both a DNA glycosylase activity and another activity that nicks DNA at AP sites. Both enzymes are metalloproteins with one zinc per molecule, and they possess a zinc finger consensus sequence at the carboxy-terminal end. Both proteins protect DNA against the mutagenic action of oxidized purines.

In *E. coli*, the *fpg* and *recA* genes are not linked (Boiteux & Huisman, 1989), and *fpg* expression is not altered by known DNA-damaging agents. In *L. lactis*, the *fpg* gene may have some other important functions. During transcription analysis of the *fpg*-L gene, we identified only an *fpg-recA* cotranscript (Duwat *et al.*, 1994), and attempts to identify an *fpg* mRNA were unsuccessful (data not shown). Furthermore, we observed that the lactococcal *recA* strain is oxygen-sensitive (Duwat *et al.*, 1994). These data led us to ask whether there may be an interaction

between the SOS-like pathway and the GO system. The recent isolation of an *L. lactis fpg* mutant may help us to understand the real function of the gene and the possible links between the different repair pathways.

Our results imply that the *fpg* gene from the Gram-positive bacterium *L. lactis* and the Gram-negative bacterium *E. coli* have issued from a common ancestor. Boiteux *et al.* (1990a) suggested the presence of Fpg proteins in other bacteria including *Salmonella typhimurium*, *Micrococcus luteus*, *Bacillus subtilis*, and our results suggest the presence of Fpg proteins in *B. firmus*, *Synechocystis* sp. and *Synechococcus* sp. The conservation of the Fpg protein in evolutionarily distant bacteria suggests that this protein plays an important role in cellular defence against free radicals and ROS attack on DNA in prokaryotic cells.

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