Molecular Microbiology (2007) **65**(2), 294–304 doi:10.1111/j.1365-2958.2007.05779.x

Involvement of a periplasmic protein kinase in DNA strand break repair and homologous recombination in *Escherichia coli*

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Summary

The involvement of signal transduction in the repair of radiation-induced damage to DNA has been known in eukaryotes but remains understudied in bacteria. This article for the first time demonstrates a role for the periplasmic lipoprotein (YfgL) with protein kinase activity transducing a signal for DNA strand break repair in *Escherichia coli*. Purified YfgL protein showed physical as well as functional interaction with pyrroloquinoline-quinone *in solution* and the protein kinase activity of YfgL was strongly stimulated in the presence of pyrroloquinoline-quinone. Transgenic *E. coli* cells producing *Deinococcus radiodurans* pyrroloquinoline-quinone synthase showed nearly four log cycle improvement in UVC dark survival and 10-fold increases in gamma radiation resistance as compared with untransformed cells. Pyrroloquinoline-quinone enhanced the UV resistance of *E. coli* through the YfgL protein and required the active recombination repair proteins. The *yfgL* mutant showed higher sensitivity to UVC, mitomycin C and gamma radiation as compared with wild-type cells and showed a strong impairment in homologous DNA recombination. The mutant expressing an active YfgL *in trans* recovered the lost phenotypes to nearly wild-type levels. The results strongly suggest that the periplasmic phosphoquinolipoprotein kinase YfgL plays an important role in radiation-induced DNA strand break repair and homologous recombination in *E. coli*.

Introduction

Mechanism of recombinational repair has been extensively studied in *Escherichia coli*, exposed to various DNA-damaging agents (reviewed in Kuzminov, 1999). Both genetic and biochemical studies have suggested that the initiation of homologous recombination follows RecBCD or the RecFOR pathways. *In vivo* (Wang and Smith, 1993) and *in vitro* (Anderson and Kowalczykowski, 1997) studies have shown that RecBCD promotes the repair of double-stranded DNA (dsDNA) breaks, whereas RecFOR is involved in the repair of single-stranded DNA (ssDNA) gaps (reviewed in Rocha *et al.*, 2005). Both the pathways provide ssDNA molecules coated with RecA to allow the invasion of a homologous molecule. Mutation in *recBC* genes confers low viability of *E. coli* unless they acquire mutation in *sbcA*, *sbcB* and *sbcC/sbcD* genes and become proficient in RecF pathway of homologous recombination and/or illegitimate recombination (Churchill *et al.*, 1999). The *recA* mutants of *E. coli* show the blocked genetic recombination (Clark and Margulies, 1965), sensitivity to DNA-damaging agents, impairment in induction of prophages, bacteriocins and SOS genes, and defect in recombinational or post-replication repair (reviewed in Smith, 2004).

Exposure of cellular DNA to UVC radiation gives rise predominantly to dimeric pyrimidine photoproducts including cyclobutane dimmers and pyrimidine (6-4) pyrimidone photoadducts (Cadet *et al.*, 2005). The levels of DNA strand breaks and 8-oxo-7, 8-dihydroguanine (8-oxoGua), the main base oxidation products, are significantly low as compared with overall bipyrimidine photoproducts. These impairments are corrected by (i) the light-stimulated photoreactivating enzyme system, (ii) excision repair, which constitutes the major component of UV dark repair (Lage *et al.*, 2003) and (iii) error-prone recombination repair mechanisms in *E. coli* (reviewed in Smith, 2004). Different genes and their products involved in DNA repair have been identified based on the response of organisms to DNA-damaging agents in appropriate genetic backgrounds (reviewed in Humayun, 1998). DNA damage induces the levels and/or activity of DNA repair proteins by still largely unknown mechanism in prokaryotes. In eukaryotes, the recruitment of DNA repair proteins to the site of DNA damage occurs through post-translational modification-mediated protein–protein interaction (Ira *et al.*, 2004).

Deinococcus radiodurans R1 (henceforth referred to as *DEIRA*), a radioresistant bacterium, confers a highly proficient DNA strand break repair mechanism and exhibits

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extreme tolerance to the lethal and mutagenic effects of ionizing and non-ionizing radiations (reviewed in Makarova *et al*., 2001) and other DNA-damaging agents (Battista, 2000). DNA strand break repair involves the reassembling of DNA fragments by an extended synthesis-dependent strand annealing (ESDSA) mechanism (Zahradka *et al*., 2006) and contributes maximally to the extreme radioresistance in *Deinoccocus*. *DEIRA* genome encodes a pyrroloquinoline-quinone (PQQ) synthesis system and synthesis of PQQ has been shown to protect heterologous bacteria from oxidative stress (Khairnar *et al*., 2003). PQQ as an antioxidant metabolite detoxifies reactive oxygen species more efficiently than other natural antioxidants (Misra *et al*., 2004). PQQ is also known to be a redox cofactor for dehydrogenases (Goodwin and Anthony, 1998) and as a member of B-group vitamins (Kasahara and Kato, 2003). PQQ-dependent dehydrogenases contain a well-conserved PQQ binding amino acid motif. Constitution of an active quinoprotein requires the synthesis of apoenzyme and PQQ, transport of these molecules to periplasm and their incorporation together with divalent ions. A large number of ser/thr kinases of bacteria including *D. radiodurans*, fungi and mammalian system show putative PQQ binding motifs. Some of these kinases may be involved in intracellular signalling using PQQ as stimulus but have not been identified.

This study reports the identification of a hitherto unknown role of the periplasmic lipoprotein, YfgL (Blattner *et al*., 1997), namely in the dark repair of UVC-, gamma radiation- and mitomycin C (MMC)-induced DNA damage in *E. coli*. Our results demonstrate that *E. coli* YfgL is a quinoprotein and a protein kinase, which can interact with PQQ *in vitro* and *in vivo* to enhance the radiation tolerance of *E. coli*. YfgL functions require *recA* and *umuDC* and contribute to DNA strand break repair possibly through a signalling mechanism. Inhibition of homologous recombination in *yfgL* mutant with wild-type *recA* further suggested the strong interaction of these two genes in genetic recombination.

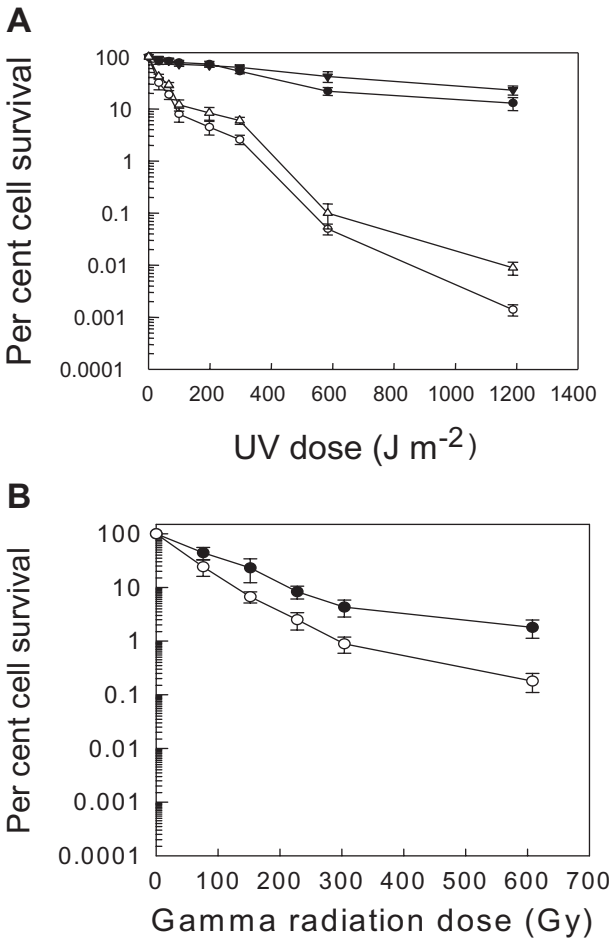

Fig. 1. Radiation response of *E. coli* cells expressing pyrroloquinoline-quinone (PQQ) synthase from *Deinococcus radiodurans*. *E. coli* strain AB1157 (▼, △) and BL21 (●, ○) cells harbouring plasmid vector (△, ○) and their respective derivatives synthesizing PQQ (▼, ●) were exposed with different doses of UVC (A) and gamma radiation (B) and cell survival was monitored. Initial cell density of cultures used in all experiments for Figs 1–5 was 10^8 cells ml^{-1}.

Results

PQQ enhances radiation tolerance in E. coli

Transgenic *E. coli* strains expressing PQQ synthase from *Deinococcus* were constructed in *E. coli* strain BL21 as described earlier (Khairnar *et al*., 2003) and a derivative of K-12, *E. coli* strain AB1157. The synthesis of PQQ in transgenic *E. coli* was ascertained indirectly by monitoring the complementation of mineral phosphate solubilization function of *E. coli* as described earlier (Khairnar *et al*., 2003) (data not included). Such cells showed more than 1000-fold higher UVC dark survival and nearly a 10-fold higher tolerance to gamma radiation, at higher doses of irradiation, as compared with respective controls (Fig. 1). The enhancement of UV tolerance in *E. coli* cells producing PQQ was observed both in *E. coli* strain B and in strain K-12 genetic background. *E. coli* protein database was searched for the PQQ binding motif-containing proteins. Results showed that two *E. coli* proteins, namely glucose dehydrogenase (*gcd*) and YfgL protein (*yfgL*), contain PQQ binding motifs. PQQ is a known component of membrane-bound gluconic acid dehydrogenase (Cozier *et al*., 1999). YfgL, which contains seven putative PQQ binding motifs and one putative ser/thr kinase domain in *E. coli* and many other bacteria (http://www.sanger.ac.uk and http://smart.embl-heidelberg.de) has not been studied for its possible interaction with PQQ and consequent phenotypes.

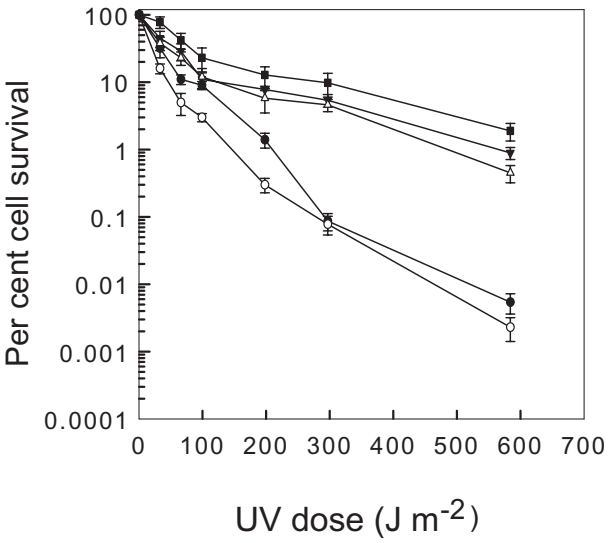

Fig. 2. Effect of *yfgL* disruption on radiation response of *E. coli* AB1157. The *E. coli* AB1157 (▼, △) and *yfgL*::*cat* mutant (●, ○) cells were exposed to different doses of UVC radiation and cell survival was monitored in light (△, ○) and dark (▼, ●) for UV experiment. UVC dark survival of mutant expressing wild-type YfgL (■) on a multicopy plasmid is also shown.

YfgL plays a role in UV radiation tolerance

The genomic copy of *yfgL* locus was disrupted with chloramphenicol acetyl transferase (*cat*) gene in *E. coli* AB1157 and BL21 strains (Fig. S1). AB1157 mutant showed a significant drop in the colony-forming units when exposed to higher doses of UV and incubated in the dark. The colony-forming units of such cells when exposed to similar doses of UV and incubated in light showed less effect as compared with dark-incubated control (Fig. 2). The UV resistance of *yfgL*::*cat* mutant also decreased significantly in strain BL21 (data not shown). Overexpression of YfgL on multicopy plasmid in *yfgL*::*cat* mutant of *E. coli* AB1157 showed the transcomplementation of UV dark survival nearly equal to wild type (Fig. 2). The results suggested that YfgL role in UV resistance was independent of photoreactivation repair.

PQQ effect on radiation response of E. coli *is linked to* yfgL *activity*

The *yfgL* mutant of *E. coli* AB1157, synthesizing PQQ was exposed to different doses of UV and gamma radiations and cell survival was monitored. Results showed similar effect of UV and γ rays on *yfgL*::*cat* mutant both in the absence (Fig. 2A) and in the presence (Fig. 3) of PQQ. These data suggested that the higher radiation tolerance mediated by PQQ requires the presence of a functional copy of *yfgL* in *E. coli*. Also that *yfgL* has an inherent role in radiation resistance of wild-type *E. coli*.

The role of YfgL in PQQ-mediated radiation stress response was further confirmed by overexpression of YfgL in *yfgL*::*cat* mutant strains concurrently with PQQ synthase from *D. radiodurans* KR1. The recombinant plasmid, pQyfg (both *pqqE* and *yfgL* genes independently under the control of T7 promoter in pET28a+), was transformed into *E. coli* BL21 (DE3) pLysS and its *yfgL*::*cat* mutant derivative and both PQQ synthase and YfgL were expressed concurrently with IPTG (data not shown). These cells showed nearly 70% restoration of PQQ stimulation of UV tolerance in *yfgL* mutant (Fig. 3A) and complete recovery of gamma radiation resistance to the wild type (Fig. 3B), supporting the finding that PQQ-induced radiation resistance was mediated through YfgL.

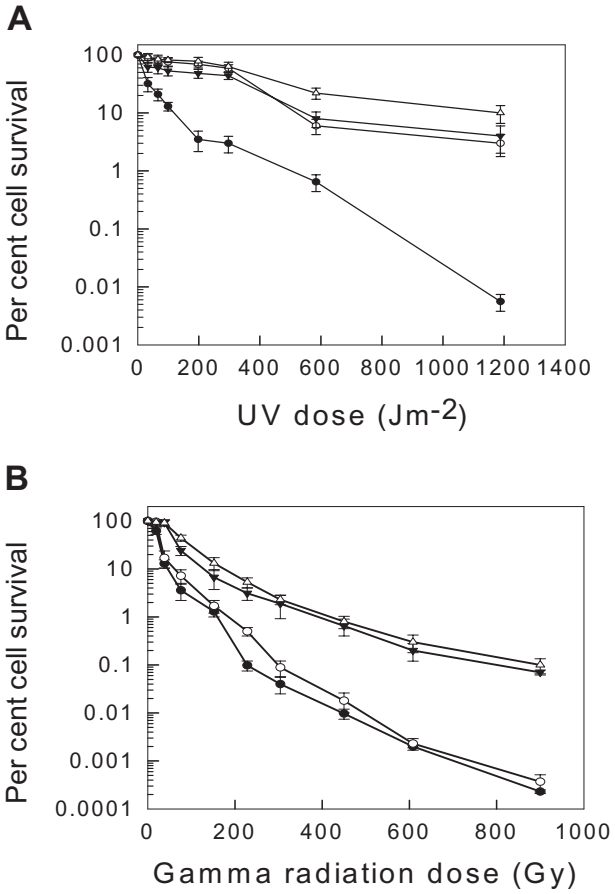

Fig. 3. Effect of *yfgL* mutation on PQQ-mediated radiation tolerance in *E. coli* BL21.
A. *E. coli* mutant (●, ○) and wild-type (▼, △) cells expressing only PQQ synthase (●, ▼) or both PQQ synthase and YfgL together (○, △) *in trans* were checked for UVC dark survival.
B. *E. coli* AB1157 (▼), its *yfgL*::*cat* mutant (●), expressing PQQ synthase (○) and wild-type YfgL on multicopy plasmid (△) were exposed with different doses of gamma radiation and cell survival was monitored.

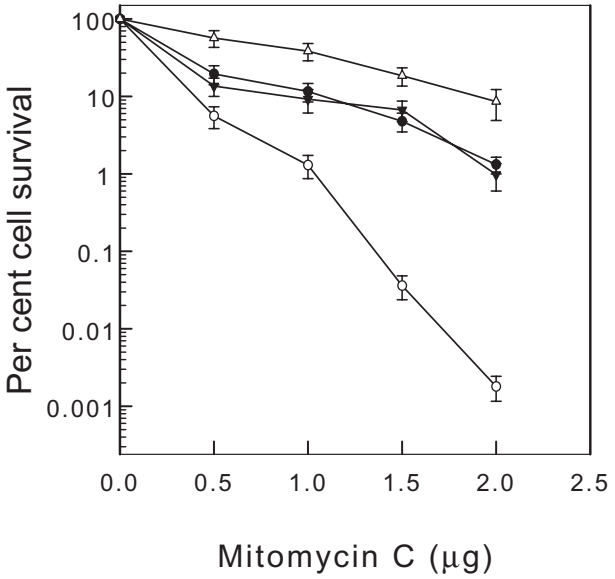

Fig. 4. Effect of mitomycin C on *E. coli* cell survival. *E. coli* AB1157 (▼), expressing PQQ synthase (△) and its *yfgL*::*cat* derivative (○) expressing wild-type YfgL on multicopy plasmid (●) were treated with different concentration of mitomycin C for 1.5 h and cell survival was monitored.

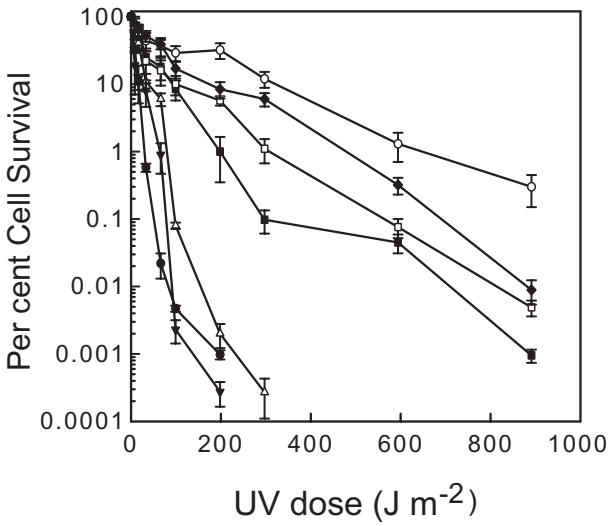

Fig. 5. Effect of PQQ synthesis on UVC response in UV repair mutants of *E. coli* AB1157. The *uvrA* (●, ○), *recA* (▼, △) and *umuD/C* (■, □) mutants of *E. coli* were transformed with plasmid vector (○, □, △) for control and pTrcpqq (●, ■, ▼) for the expression of PQQ, and exposed with different doses of UVC radiation. The dark cell survival was compared with AB1157 (■) as wild-type control. Cells were irradiated with UVC at a dose rate of 1.23 J s^{-1} m^{-2}.

YfgL is required for MMC tolerance in E. coli

Effect of MMC on cell survival was monitored in *E. coli* AB1157 expressing PQQ synthase and carrying mutation in *yfgL* gene. The *yfgL*::*cat* mutant showed higher sensitivity as compared with wild-type control (Fig. 4). Overexpression of multicopy wild-type YfgL in *yfgL*::*cat* mutant background restored the wild-type tolerance for MMC. Wild-type cell expressing PQQ synthase showed nearly 10-fold higher tolerance as compared with control. The result suggested that *yfgL* also plays an important role in MMC-induced DNA damage repair in *E. coli*.

YfgL contributes to UVC tolerance possibly through recombination repair

In order to trace the pathway downstream to YfgL, the effect of PQQ in UV-sensitive mutant strains of *E. coli* having wild-type copy of YfgL was further studied. PQQ synthase was expressed in *uvrA* (nucleotide excision repair mutant), *umuD* and *recA* (recombination repair mutant) and the effect of PQQ on UV tolerance was monitored in these genetic backgrounds. The *uvrA* mutant showed PQQ-mediated enhanced UV dark survival as compared with control but the stimulatory effect of PQQ on UV dark survival was not observed in *umuDC* and *recA* minus cells (Fig. 5). Thus, functional UmuDC and RecA were needed for the expression of PQQ-mediated UV tolerance phenotype.

YfgL is required for homologous recombination

Conjugal crosses were made between *HfrC* and *E. coli* XL-1 Blue as donor and AB1157, *yfgL*::*cat* mutant and their PQQ synthese-expressing derivatives as recipients. The *yfgL*::*cat* mutant showed severe loss of homologous recombination proficiency of wild-type *E. coli* (Table 1) while the F′ transfer proficiency was nearly similar to that of wild-type cells. PQQ effect observed in wild-type cells was absent in *yfgL*::*cat* mutant. Similar results were observed with the auxotrophic markers, scored in independent studies (data not shown). Zygotic induction data clearly ruled out any defect in the transfer of genetic

Table 1. Genetic transformation and recombination proficiencies of wild type and *yfgL*::*cat* mutant of *Escherichia coli* with and without expressing pyrroloquinoline-quinone synthase.

Recipient strains	Ara$^+$ Str^r recombinants[a]	Tet^r Str^r recombinants[b]
AB1157	1.0	1.0
AH1157	0.001	0.87
AB1157 + pTrcpqq	1.33	0.97
AH1157 + pTrcpqq	0.0018	0.93

a. Wild-type frequency of 1.0 corresponds to 5 transconjugants per 100 *HfrC* cells.
b. Wild-type frequency of 1.0 corresponds to 12 transconjugant per 100 F′ cells.
Conjugational crosses were carried as described in *Experimental procedures*. Experiments have been repeated at least three times. The values given are means of triplicate from a typical representative experiment where differences among replicates were less than 15%.

Table 2. Zygotic induction of prophage after transfer by conjugation.

Recipient strains	Per cent frequency of zygotic induction per recipient ± SD
AB1157	4.92 ± 0.37
AH1157	3.36 ± 0.89

Experiments have been repeated at least three times and data presented are the average results with standard deviation (SD) from all three experiments.

materials into *yfgL*::*cat* mutant strain of *E. coli* (Table 2). The results strongly suggest the role of YfgL in homologous recombination.

PQQ binds YfgL and stimulates autophosphorylation in solution

The YfgL contains a putative ser/thr kinase domain. Purified recombinant YfgL from *E. coli* BL21 (Fig. 6A) was used for monitoring its interaction of PQQ and consequences for kinase function. Immunoblotting of recombinant protein in the absence and presence of ATP, with monoclonal antibodies against phospho-ser/thr epitopes, indicated that YfgL carries a phosphoryl group at ser/thr amino acids in its primary structure (Fig. 6A). Alkaline phosphatase treatment of YfgL abolishes the cross-reactivity to antibodies. Dephosphorylated protein when incubated with ATP regained the cross-reactivity with antibodies. The phosphorylation by ATP was significantly enhanced in the presence of PQQ, establishing the PQQ-stimulated protein kinase activity of YfgL.

PQQ physically interacts with YfgL

Pyrroloquinoline-quinone interaction with YfgL was ascertained by determining absorption spectra of (i) free PQQ, (ii) PQQ incubated with YfgL and then unbound PQQ removed from mixture, and (iii) only the YfgL protein. The YfgL incubated with PQQ showed a broad peak at λ_{max} 330 nm, which was also seen in the reference spectrum of PQQ and has been reported earlier (Boling and Setlow, 1966). The 330 nm peak was absent in YfgL sample and in reference PQQ when passed through Sephadex G-25 (Fig. 6B). The results clearly showed that PQQ interacts with YfgL in a chemical environment which does not truly mimic the biomembranes. Nonetheless, YfgL showed the characteristics of a quinoprotein *in solution*. The stimulation of autophosphorylation activity of YfgL protein by PQQ (Fig. 6) demonstrates the structural as well as functional nature of this interaction.

Multicopy effect of YfgL on host gene expression

Escherichia coli BL21 cells overproducing YfgL showed induction of at least six other proteins. Some of these proteins were also induced on overexpression of PQQ synthase in wild-type *E. coli* cells (Fig. 7). The result suggested that overexpression of YfgL and/or PQQ synthase in the presence of native allele of *yfgL* induces the expression of certain unidentified proteins in *E. coli.*

Discussion

The present study has described the role of a periplasmic lipoprotein YfgL, in stress-related signal transduction in

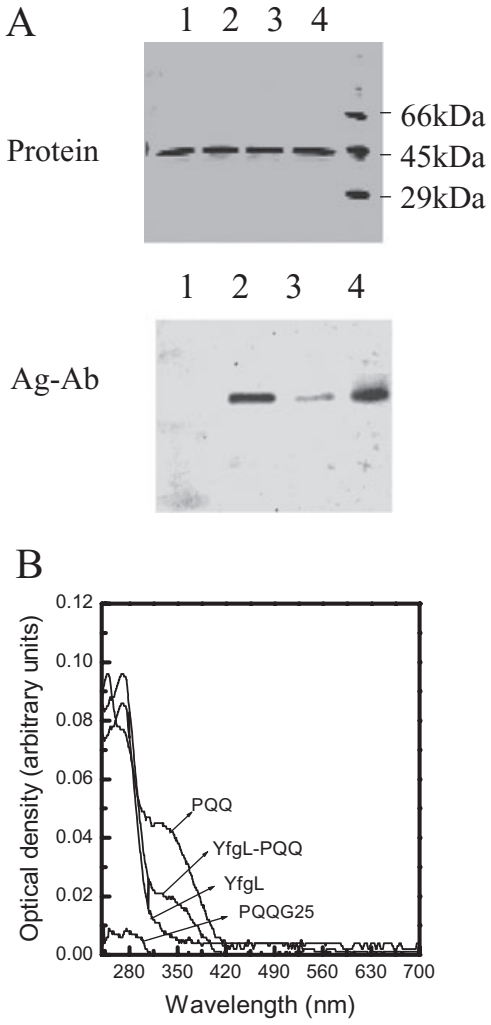

Fig. 6. Recombinant YfgL interacts with PQQ and undergoes autophosphorylation.
A. Approximately 100 ng of near homogenous YfgL protein was dephosphorylated with alkaline phosphatase (1) and then incubated with 5 mM ATP in the absence (3) and presence (4) of 1 μM PQQ at 37°C for 30 min. Treated proteins along with purified recombinant YfgL (2) were analysed on SDS-PAGE and immunoblotted with phospho-ser/thr antibodies.
B. Absorption spectra of YfgL incubated with PQQ and unbound PQQ was removed through Sephadex G-25 (YfgL–PQQ), purified recombinant protein (YfgL) were compared with absorption spectra of PQQ as such (PQQ), and PQQ passed through Sephadex G-25 column (PQQG25).

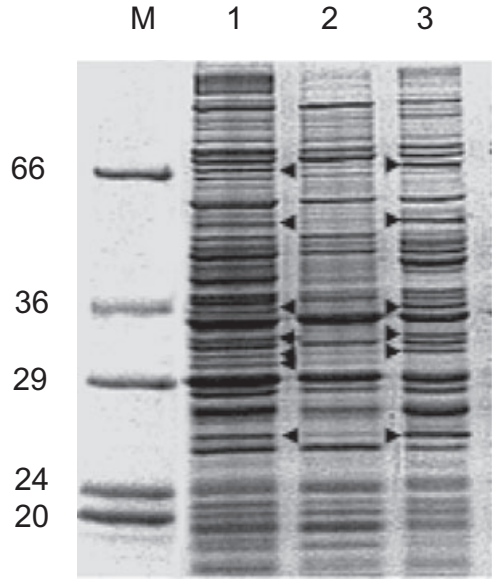

Fig. 7. Protein profiles of *E. coli* cells expressing YfgL and PQQ synthase on multicopy plasmids. *E. coli* BL21 harbouring pETyfgL (1), pET28a+ (2) and pETpqq (3) were induced with IPTG and total proteins were analysed on 10% SDS-PAGE. The protein sizes were determined using standard protein molecular weight marker (M).

E. coli. The protein has been characterized as a quinoprotein kinase and shown to play a role in UV dark recombination repair in *E. coli*. The *yfgL* contribution in recombination repair of DNA strand break was triggered by our observation that deinococcal PQQ synthase expression in *E. coli* enhanced the tolerance to DNA-damaging agents like UVC, gamma radiation (Fig. 1) and MMC (Fig. 4). Additional evidence for *yfgL* role came from following observation: (i) enhanced UVC survival requires wild-type *yfgL*, and (ii) YfgL interaction with PQQ, which also stimulates autophosphorylation activity of recombinant YfgL *in solution*.

UVC causes intrastrand cross-links in DNA template (Rupp and Howard-flanders, 1968). Intrastrand cross-linking of DNA causes the production of daughter strand gaps (DSGs) due to reinitiating of replication downstream of cross-links. Replication stalled or distorted at cross-links in template DNA might result in double-stranded breaks in one of the daughter strands which acts as substrate for recombinational repair (reviewed in Kuzminov, 1999). Gamma radiation exposure produces both single-strand and double-strand breaks in dose-dependent manner. MMC induces interstrand cross-links leading to double-strand breaks (Kumar *et al*., 1997). Involvement of both RecBCD and RecFOR recombination pathways in the repair of UV-, gamma radiation- and MMC-induced DNA damage has been shown in *E. coli* (Keller *et al*., 2001). Our results showed that YfgL is required for UV, gamma radiation and MMC tolerance in *E. coli* and *yfgL* mutant was also severely impaired in conjugal recombination. Near to complete inhibition of genetic recombination (Table 1) and sensitivity of *yfgL*::*cat* mutant of *E. coli* to DNA-damaging agents were very similar to *recA* null mutants of *E. coli* (Clark and Margulies, 1965). As *yfgL*::*cat* mutant cells were not impaired for conjugal DNA entry (Tables 1 and 2), the inhibition of homologous recombination was more likely at the level of DNA integration in the host chromosome. YfgL might regulate the expression/activity of important recombination gene(s) whose product(s) are required during the crucial steps in homologous recombination. The results presented in this study clearly demonstrate the involvement of a membrane protein kinase YfgL, in recombination repair of DNA strand break.

The precise mechanism of YfgL action is not yet known. YfgL has been shown to contribute to (i) invasive ability and virulence of *E. coli* strain LF82 (Rolhion *et al*., 2005), (ii) cold adaptation in wild-type *E. coli* (Phadtare and Inouye, 2004), and (iii) membrane biogenesis and selective transport of metabolites across membrane (Ruiz *et al*., 2005; Wu *et al*., 2005). These results indicate the possible global role of this protein in response to abiotic stresses. YfgL was recently shown to exist in a multiprotein complex with YaeT, YfiO and NlpB, which are involved in membrane biogenesis (Wu *et al*., 2005) and regulates selective transport of small size molecules across cell membrane (Ruiz *et al*., 2005). YfgL is expressed under the control of sE, a positive regulator of several membrane-located stress-responsive genes (Onufryk *et al*., 2005). In *Bacillus subtilis*, YxaL (a structural orthologue of YfgL protein in *E. coli*) has been shown to enhance the processivity of PcrA (UvrD in *E. coli*) helicase *in vitro* (Noirot-Gros *et al*., 2004). One way YfgL can control such diverse processes is by acting as a common transducer of the different stress-responsive signals and regulating the expression of various downstream proteins involved in specific responses.

Recently it has been shown that YfgL is an outer membrane lipoprotein and its amino-terminal lipid moiety likely embeds the protein in the bilayer with the bulk of the protein remaining in the periplasm (Wu *et al*., 2005), which resembles the organization of membrane proteins acting as transducers. The results reported in Figs 2, 3 and 6 suggest that the observed PQQ effects were through YfgL. The *yfgL* is part of an operon, with *yfgM* upstream and *der* downstream, under *yfgM* promoter in *E. coli*, and hence the *yfgL*::*cat* phenotype could have arisen due to the absence of either YfgL or product of downstream ORF. The complementation of *yfgL* phenotype in mutant expressing YfgL protein (Figs 2, 3B and 4) showed that PQQ-induced enhancement of UV resistance needs a functional YfgL (a quinolipoprotein kinase) which undergoes autophosphorylation *in solution*. Membrane location and autophosphorylating activity of YfgL allow it to be an appropriate

component of signal transduction, which may use PQQ as an inducer. *E. coli* does not encode for PQQ synthesis and in *E. coli* YfgL may interact with other inducers yet to be identified. However, in bacteria which posses both YfgL and PQQ, the later may provide the necessary signal for YfgL-based transduction. The phosphorylation of another periplasmic protein involved in signal transduction in response to nutritional stress has been shown earlier (Celis *et al*., 1998).

The role of signal transduction in recombination repair of DNA damaged by ionizing radiation and non-ionizing radiations has not been given enough attention in prokaryotes. Recently, the tyrosine phosphorylation of single-stranded DNA-binding protein (SSB), a recombination/repair protein, has been reported for the first time in bacterial systems (Mijakovic *et al*., 2006). The authors have demonstrated that SSB phosphorylation enhances its ssDNA binding activity by a factor of 200 when compared with the non-phosphorylated protein. Furthermore, besides DNA strand break repair, the UV radiation-induced expression of JNK and NFkappaB signal transduction pathways proteins have also been reported in mammalian cells (Adachi *et al*., 2003). The possible involvement of phosphorylation/dephosphorylation mechanism of YfgL in the regulation of downstream synthesis of other proteins required for UV dark repair function seems quite likely. SDS-PAGE analysis of total proteins of *E. coli* BL21 expressing YfgL on a multicopy plasmid showed the upregulation of six proteins (Fig. 7), some of which were also induced upon overexpression of PQQ synthase in *E. coli* cells harbouring wild-type *yfgL* allele. Whether or not these proteins are under the regulatory control of YfgL and have a role in DNA repair is yet to be ascertained.

YfgL regulation of PQQ-mediated UV resistance in *E. coli* strongly supported the concept of PQQ acting as an inducer for a protein involved in UV dark repair. PQQ acts as a redox cofactor for enzymes required for mineral phosphate solubilization in bacteria (Goodwin and Anthony, 1998) and for other biochemical processes in eukaryotes (Aizenman *et al*., 1994; Wang *et al*., 2005) including lysine metabolism (Kasahara and Kato, 2003). PQQ protects the mitochondrial activity from oxidative stress and as an essential nutrient for normal growth of animals (He *et al*., 2003; Stites *et al*., 2006) and bacterial cells from photodynamic killing by rose bengal (Khairnar *et al*., 2003). However, the mechanisms of PQQ action at cellular and molecular levels have not been understood. Protein database search for PQQ binding amino acid motifs has indicated a large number of proteins across the prokaryotic and eukaryotic organisms, with putative ser/thr kinases domain and having PQQ binding motifs.

The findings from this study provide new insights into the understanding of the basic mechanism of DNA repair in bacteria These include the (i) involvement of signal transduction mechanisms in the recombination repair of radiation-induced DNA strand break in prokaryotes also, and (ii) PQQ, as a cofactor for membrane bound/soluble ser/thr kinases which has a role in repair of radiation-induced DNA damage. By using PQQ as a probe, the identification and characterization of unknown protein kinases and their role in various physiological and biochemical processes including DNA recombination repair can be explored effectively.

Experimental procedures

Bacterial strains, plasmids and molecular biology reagents

Bacterial strains and plasmids used in this study are given is Table 3. *E. coli* cells harbouring pET28a+, pBluescript SK+ and pTrc99 and their respective derivatives were grown in the presence of appropriate antibiotics at 37°C with constant shaking at 180 r.p.m. All the chemicals of molecular biology grade were obtained from Sigma Chemical (St Louis, MO), Bethesda Research Laboratory (Bethesda, MD) and Sisco Research Laboratory (Mumbai, India). The restriction enzymes and DNA modifying enzymes were obtained from Roche Molecular Biochemicals (Mannheim, Germany), New England Biolabs (Ipswich, MA) and Bangalore Genei (Bangalore, India).

Construction of expression plasmids and inducible expression of recombinant protein

The *pqqE* gene was cloned in pET28a+ to yield pETpqq as described earlier (Khairnar *et al*., 2003) and at EcoRI and BamHI sites of pTrc99 to yield pTrcpqq using primers PRN1 (5′-CGGAATTCATGGTGGCATTTCTCCGT-3′) and PRN2 (5′-CGGGATCCTCAATGCGT GACTTACCA-3′). The 1.1 kb fragment of *yfgL* was PCR amplified using PRN3 (5′-CGGAATTCATGCAATTGCGTAAATTACTGCTGCC3′) and PRN4 (5′GCCAAGCTTTCAGACAACGCACGCTATAT TCG-3′) gene-specific primers. A EcoRI–HindIII fragment of PCR product was cloned at compatible ends in pTrc99 and pET28a+ to yield pTrcyfg and pETyfg respectively. The *yfgL* tagged with T7 promoter was PCR amplified from pETyfgL using PRN5 (5′-CGGCGTAGAGGATCGAGAT-3′) and PRN4 primers and was subcloned at end-filled site of XhoI in pETpqq to yield pQyfg. The expression of recombinant proteins from pETpqq, pETyfgL and pQyfg was checked in *E. coli* BL21 (DE3) pLysS and its derivatives, and from pTrcpqq, pTrcyfg was monitored into *E. coli* JM105/AB1157 and its derivatives. Inducible expression of recombinant protein was carried out as described earlier (Sambrook and Russell, 2001). The vector controls pET28a+ and pTrc99 were used for appropriate comparison in respective *E. coli* hosts. The synthesis of recombinant proteins was monitored by SDS-polyacrylamide gel electrophoresis as described earlier (Laemmli, 1970).

Table 3. List of bacterial strains and plasmids used.

Bacterial strains/plasmids	Relevant characteristics	Source
Bacterial strains		
Deinococcus radiodurans KR1	Wild type	Narumi *et al.* (2001)
E. coli AB1157	*Thi*$^{-1}$,*thr*$^{-1}$,*leuB6,*Δ*(gpt-proA)62,hisG4,argE3,lacY,galk, mtl*$^{-1}$,*rpsL31,kdgK-51,tsx-33,gsr′-0,glnV44,Lam-, Rac-0,rfbC1,ropoS396,xylA5*	Laboratory collection
E. coli BL21 (DE3) pLysS	*F*$^{-}$, *ompT*, *gal[dcm], [lon], hsdS*$_B$ (*r*$^{-}_B$ *m*$^{-}_B$, an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene, pLysS	Laboratory collection
E. coli MD2223	*HfrC*, *relA1 tonA22*, T2 resistant	Laboratory collection
E. coli MD17671	*HfrH*, *deoC2 trypR1*, λ lysogen Strp^{S}	Laboratory collection
E. coli MD2042	AB1157, *recB156, recC22, sbcB15, sbcC201*	Laboratory collection
AB1886	AB1157, *uvrA6*	Laboratory collection
GY8347	AB1157, *F*$^{-}$ Δ*lac-pro,* Δ*umuDC*	Sommer *et al.* (1993)
JC1553	*leuB6,hisG4,argE3,rpsL31,met*$^{-}$,*recA1*	Clark and Margulies (1965)
AH1157	AB1157, *yfgL*::*cat*	This study
HL21	BL21, *yfgL*::*cat*	This study
Plasmids		
pET28a+	5.369 kb, Kan^{R}	Novagen
pTrc99A	4.176 kb, Amp^{R}, Accession No. U13872	Pharmacia
pSK+	2.961 kb, Amp^{R}, Accession No. X52328	Stratagene
pETpqq	6.524 kb, *pqqE* (D.r) in pET28a^{+}	Khairnar *et al.* (2003)
pETyfg	6.503 kb, *yfgL* in pET28a^{+}	This study
pQyfg	8.0 kb, 1481 bp, *PT7-yfgL* of pETyfg at filled XhoI site in pETpqq	This study
pTrcyfg	5.310 kb, *yfgL* in pTrc99A	This study
pTrcpqq	5.331 kb, *pqqE*(D.r) in pTrc99A	This study
pSKyfg:cat	4.885 kb, *yfgL*::*cat* at *SmaI* in pSK+	This study

Preparation of insertional mutants of E. coli

Insertional inactivation of *yfgL* gene in *E. coli* chromosome was carried out by using the standard recombinant DNA techniques. In brief, the *yfgL* gene was PCR amplified using gene-specific primers from genomic DNA of *E. coli* W3110. The chloramphenicol acetyl transferase (*cat*) gene was PCR amplified using gene-specific primers PRN6 (5′-GTTAACTGACGGAAGATCACTTCGCA3-′) and PRN7 (5′-GTTAACCACACGGTACATTGCTTCC-3′) from *E. coli* genome harbouring Tn*9*. The *yfgL* gene (1134 bp) was digested with HpaI and ligated with HpaI-digested PCR product of *cat* gene. The *yfgL*::*cat* cassette was PCR amplified from ligated mixture using *yfgL*-specific flanking primers. The presence of *cat* gene in 2.1 kb product was further confirmed using internal *cat* primers. The amplified 2.1 kb DNA fragment having inserted *cat* gene in *yfgL* DNA was cloned at SmaI site in pBluescriptSK+ to yield pSKyfg:cat (Fig. S1A). Plasmid was linearized with PvuI and transformed into *E. coli* MD2042 (*recB*$^{-}$ and *sbcB*$^{-}$ mutant strain) and transformants were scored on LB agar plate containing chloramphenicol (15 μg ml^{-1}). Replacement of chromosomal copy of *yfgL* with *yfgL*::*cat* cassette in chromosome was confirmed by PCR amplification of *yfgL*::*cat* using *yfgL*-specific primers and internal *cat* primers (Fig. S1B). The *yfgL*::*cat* disruption was transduced into AB1157 and BL21 chromosome independently by P1*vir*-mediated generalized transduction as described earlier (Miller, 1992).

Cell survival studies

Escherichia coli cells harbouring recombinant plasmids and corresponding vectors were induced with 200 μM IPTG for 3 h and irradiated with different doses of UV at 254 nm at dose rate 0.295 J s^{-1} m^{-2} (Laser Power Meter Gentec, Ontario, Model PSV-3303) (as described by Kota and Misra, 2006) and gamma radiation using Cobalt 60 at the dose rate 6.83 Gy min^{-1} (Gamma Cell 220) (as described by Misra *et al.*, 2006). For allowing UV repair in the dark, the plates were wrapped with aluminium foils and incubated in the dark at optimum conditions. For monitoring the effect of light on UVC survival, the irradiated cells were illuminated with fluorescent tube light for 45 min and plates were incubated at 37°C in the dark. MMC effect on cell survival of *E. coli* was monitored as described earlier (Keller *et al.*, 2001).

Conjugal cross and zygotic induction studies

Escherichia coli AB1157, its *yfgL*::*cat* mutant and their *pqqE*-expressing transgenic derivatives were used as recipients in conjugal crosses as described by Miller (1992). Arabinose (Ara^{+}) recombinants were scored for conjugal crosses with *HfrC* (*E. coli* MD2223) (Mangoli *et al.*, 1997). F′ transfer was performed using *E. coli* XL-1 Blue (New England Biolab, Beverly, MA) as donor and the transconjugants were scored on LB plates containing tetracycline 12.5 μg ml^{-1} and streptomycin 100 μg ml^{-1}. The transconjugants obtained from *pqqE*-expressing recipient cells were scored in the presence of IPTG (500 μM) and ampicillin (100 μg ml^{-1}) while AB1157 harbouring pTrc99 served as control. Controls data were normalized for the calculation of recombination and transfer frequencies of *pqqE*-expressing transconjugants.

Zygotic induction studies were carried out as described earlier (Wood and Egan, 1981). In brief, the overnight grown cultures of donor *E. coli* MD17671 [HfrH (λ)] and recipients were diluted and grown to mid-exponential phase before they

were mixed in 1:10 ratio of donor to recipient. After 5 min of incubation, the mating was disrupted by 100-fold dilution following by agitation. Appropriate dilutions were mixed with indicator bacteria and plated on LB plates containing 10 mM $MgSO_4$, 5 mM $CaCl_2$ and streptomycin (150 μg ml^{-1}). Numbers of plagues were counted and per cent frequency of zygotic induction per recipient was calculated.

Purification and characterization of YfgL for PQQ binding and protein kinase activity

Escherichia coli expressing YfgL under the control of T7 promoter was induced with 0.5 mM IPTG and recombinant protein was purified using Ni-NTA agarose chromatography as described earlier (Misra *et al*., 1998). For PQQ binding activity assay of YfgL, the purified protein was incubated with 70 molar excess ratio of PQQ in sample buffer containing 4 M Urea and dialysed overnight at 10°C. Free PQQ was removed using Sephadex G-25 spin columns (GE Healthcare). Equal amount of free PQQ was passed through the spin columns for ascertaining the complete trapping of unbound PQQ in G-25 matrices. Free PQQ, YfgL and PQQ and YfgL–PQQ mixture passed through G-25 spin columns were used for recording the absorption spectra as described earlier (Misra *et al*., 2004). The protein kinase activity of purified YfgL was assayed by monitoring the autophosphorylation of phosphatase-treated YfgL with 5 mM ATP *in solution*. In brief, protein was incubated with alkaline phosphatase at 37°C for 1 h (Mishra and Parnaik, 1995). After phosphatase inactivation with 10 mM sodium fluoride, alkaline phosphatase-treated protein was incubated with or without PQQ (1 μM) at 37°C for 1 h in a reaction mixture containing 10 mM Tris-HCl, pH 7.6, 20 mM KCl, 0.5 mM DTT, 2% Glycerol, 1.5 mM $MgCl_2$ and 5 mM ATP. Reaction was terminated with equal volume of 2× SDS-PAGE sample buffer (Laemmli, 1970) and heating at 95°C for 5 min. For determining the autophosphorylation of YfgL with ATP and effect of PQQ on its activity, samples were separated on SDS-PAGE, transferred to PVDF (Misra and Mahajan, 2000) and immunodetected blotted with antibodies against phospho-ser/thr epitopes (Cell Signalling Technology, USA) by following manufacturer's protocols. Blots were developed using Luminol chemiluminescence (Roche Biochemicals, Mannheim) as described in manufacturer protocols and gel was documented (SYNGENE, UK).

Data presented without standard deviations are illustrative of a typical experiment and represent the average of three replicates wherein the variation among replicates was less than 15%. All experiments were repeated at least three times.

Acknowledgements

Authors are thankful to Dr (Mrs) V.P. Joshi for her critical comments on data interpretation during the preparation of manuscript and Professor I. Narumi, Japan and Professor Suzanne Sommer, Orsay, Cedex, France, for generous gifts of *D. radiodurans* KR1 and *E. coli* strain GY8347 respectively. V.A.K. thanks DAE for the award of a research fellowship.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. Insertional inactivation of *yfgL* in *E. coli* BL21 and AB1157.
A. Partial restriction map of plasmid construct having *cat* inserted at HpaI site flanking *yfgL* at 587 bp into two halves (*yfgL1* and *yfgL2*) and cloned at pBluescriptSK+.
B. PCR characterization of the *yfgL*::*cat* mutant of BL21 strain of *E. coli*. Genomic DNA of recipient host (2, 3) and transductant (4, 5) was used for PCR amplification with *yfgL* (2, 4) and *cat* (3, 5) specific primers. Lane 1 and 6 represent the molecular size markers as HindIII and EcoRI digest of λ DNA and HaeIII digest of ϕX174 respectively.

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