

DNA Gyrase of *Deinococcus radiodurans* is characterized as Type II bacterial topoisomerase and its activity is differentially regulated by PprA in vitro

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Abstract The multipartite genome of *Deinococcus radiodurans* forms toroidal structure. It encodes topoisomerase IB and both the subunits of DNA gyrase (DrGyr) while lacks other bacterial topoisomerases. Recently, PprA a pleiotropic protein involved in radiation resistance in *D. radiodurans* has been suggested for having roles in cell division and genome maintenance. In vivo interaction of PprA with topoisomerases has also been shown. DrGyr constituted from recombinant gyrase A and gyrase B subunits showed decatenation, relaxation and supercoiling activities. Wild type PprA stimulated DNA relaxation activity while inhibited supercoiling activity of DrGyr. Lysine133 to glutamic acid (K133E) and tryptophane183 to arginine (W183R) replacements resulted loss of DNA binding activity in PprA and that showed very little effect on DrGyr activities in vitro. Interestingly, wild type PprA and its K133E derivative continued interacting with GyrA in vivo while

W183R, which formed relatively short oligomers did not interact with GyrA. The size of nucleoid in PprA mutant ($1.9564 \pm 0.324 \mu\text{m}$) was significantly bigger than the wild type ($1.6437 \pm 0.345 \mu\text{m}$). Thus, we showed that DrGyr confers all three activities of bacterial type IIA family DNA topoisomerases, which are differentially regulated by PprA, highlighting the significant role of PprA in DrGyr activity regulation and genome maintenance in *D. radiodurans*.

Keywords *Deinococcus* · DNA gyrase · Genome maintenance · PprA · Radioresistance

Introduction

Deinococcus radiodurans is a radioresistant bacterium that is known for its efficient DNA double strand break (DSB) repair (Zahradka et al. 2006; Ishino and Narumi 2015) and an extraordinary tolerance to oxidative stress (Daly et al. 2010; Tian and Hua 2010; Misra et al. 2013). It contains a multipartite genome system comprising of chromosome I, chromosome II and plasmids. Each of these genome elements are present in multiple copies (Minton 1994) and packaged in the form of a doughnut shaped toroidal structure (Levin-Zaidman et al. 2003). A number of proteins including DNA topoisomerases have been found associated with the nucleoid of this bacterium (de la Tour et al. 2013). In *Escherichia coli*, DNA topoisomerases roles in maintaining correct DNA topology as well as in the resolution of duplicated intertwined circular chromosome have been reported (Buck and Zechiedrich 2004). It has been shown that DNA topoisomerase IV (TopoIV) but not DNA gyrase is responsible for decatenation of site-specific recombination intermediates in *E. coli* chromosome (Zechiedrich et al. 1997). More recently, it is observed that

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Topo IV is actually knots and unknots the sister duplexes of DNA during replication in *E. coli* (Lopez et al. 2012). The mechanisms underlying the regulation of mutually incompatible (knot and unknot) activities of TopoIV in vivo are not known yet. The *D. radiodurans* genome does not encode TopoIV but encodes DNA topoisomerase IB (DraTopoIB) and DNA gyrase (DrGyr) (White et al. 1999). PprA (named after pleiotropic protein promoting DNA repair) has been known for its role in radiation resistance in *D. radiodurans* (Narumi et al. 2004). Earlier, PprA was shown to contribute in DSB repair by stimulating DNA ends joining activities of T4 DNA ligase, *E. coli* DNA ligase and a deinococcal DNA ligase (LigB) (Narumi et al. 2004; Kota et al. 2010). PprA and DraTopoIB were found in a multiprotein DNA processing complex characterized from this bacterium (Kota and Misra 2008). Molecular mechanisms underlying the packaging of multipartite genome in toroidal structure, its remodeling during replication, transcription, recombination and segregation, and faithful inheritance are largely unexplored in *D. radiodurans*. Recently, the identification of centromeric sequences in the chromosome I of *D. radiodurans* and functional characterization of its partitioning proteins has been reported (Charaka and Misra 2012). Also, the interaction with DNA gyrase A subunit (GyrA) and DraTopoIB with PprA and the stimulation of DraTopoIB activity by this protein have been demonstrated (Kota et al. 2014a, b). Therefore, the involvement of deinococcal DNA topoisomerases and their possible interaction with PprA in the maintenance of multipartite genome structure and functions would be worth understanding. Here, we report the functional characterization of recombinant DNA gyrase of *D. radiodurans*. Using purified recombinant gyrase A and gyrase B (GyrB) subunits of *D. radiodurans* an active DNA gyrase enzyme was reconstituted. Interestingly, DrGyr showed all three activities like DNA relaxation, supercoiling and decatenation of bacterial type IIA family DNA topoisomerases. PprA and its DNA binding mutant K133E interacted with GyrA. However, W183R mutant that had compromised both DNA binding and polymerization functions of PprA did not interact with GyrA. The relaxation activity of DrGyr was stimulated by wild type PprA but not by K133E and W183R derivatives. Interestingly, the DNA supercoiling activity of DrGyr was inhibited in the presence of wild type PprA but not mutants. These results suggested that DrGyr seems to be a sole multifunctional type IIA family DNA topoisomerase in *D. radiodurans*, and the DNA binding characteristic of PprA is crucial for its regulation of DrGyr activities at least in vitro. The possibility of DrGyr and PprA interaction contributing in dynamic regulation of the chromosome functions during growth of this bacterium may be suggested.

Materials and methods

Bacterial strains and plasmids

D. radiodurans R1 (ATCC13939) was a gift from Professor J. Ortner, Germany (Schaefer et al. 2000) and is maintained in TGY (0.5 % Bacto Tryptone, 0.3 % Bacto Yeast Extract, and 0.1 % glucose) medium at 32 °C. *E. coli* strain DH5 α is used for maintaining cloned genes on plasmids while *E. coli* BL21 (DE3) pLysS is used for the expression of recombinant protein. *E. coli* BL21 cells containing pET vectors (Novogen Inc.) and their derivatives while *E. coli* BTH101 containing pKNT25 and pUT18 plasmids (Karimova et al. 1998) and their derivatives were grown in LB broth with appropriate antibiotics. All recombinant techniques used in this study were as described earlier (Sambrook and Russell 2001).

Generation of PprA mutants by random mutagenesis

The *pprA* gene was mutagenized by error-prone PCR or hydroxylamine treatment. For error-prone PCR, the *pprA* gene was amplified using a GeneMorph PCR Mutagenesis Kit (Stratagene, Inc) with a set of primers that were used to generate the *pprA* expression plasmid pET3pprAwt (Narumi et al. 2004). For hydroxylamine treatment, pET-3pprAwt was treated with 0.1 M potassium phosphate (pH 6.0) containing 0.4 M hydroxylamine and 5 mM EDTA at 68 °C for 1 h, and purified using a MinElute Reaction Cleanup Kit (Qiagen, Inc). The purified plasmids were transformed into *E. coli* BMH 71-18 to efficiently fix mutations. Plasmids were digested with *Nde*I and *Bam*HI and mutated inserts were ligated into pET3a at *Nde*I and *Bam*HI site, and introduced into *E. coli* BL21 (DE3) to select mutant clones. Large colonies grown on LB agar plates were selected as candidate clones containing a mutation in the *pprA* gene. The presence of mutation was checked by DNA sequencing and *pprA* A397G (K133E) and *pprA* T547A (W183R) mutants have been used in this study.

Construction of expression plasmids

The ORF DR_1913 (*gyrA*) encoding GyrA and DR_0906 (*gyrB*) GyrB subunits of DrGyr in the *D. radiodurans* genome were PCR amplified from genomic DNA using the gene specific primers *gyrA*F (5' CGGGATCCATGACCGG AATCAACCT 3') and *gyrA*R (5' AGCAAGCTTTTACAG CTCGTCTTCCTTGCGA 3') for the *gyrA*, and *gyrB*F (5' C GGGATCCATGAGCTTTTCCCATGC 3') and *gyrB*R (5' A GCAAGCTTTCAGACGCTGATTTTCAGCGA 3') for the *gyrB*, respectively. PCR amplified *gyrA* (2439 bp) and *gyrB* (1992 bp) were cloned in pET28a+ vector at *Bam*HI and *Hind*III sites to generate recombinant plasmids pETgyrA

and pETgyrB. The PprA expression plasmids were constructed as described earlier (Narumi et al. 2004; Kota and Misra 2006). The coding sequences of DNA binding mutants K133E and W183R of *pprA* were cloned in pET28a+ vector at *Bam*HI and *Hind*III sites and recombinant plasmids pETK133E and pETW183R were generated. These plasmids were transferred into *E. coli* BL21 (DE3) pLysS and the recombinant cells were induced with 200 μ M IPTG. The expression of recombinant proteins was ascertained on SDS-PAGE and native PAGE.

Protein purification

Recombinant PprA and its derivatives were purified from recombinant *E. coli* expressing these proteins separately, as described earlier (Kota and Misra 2006). Recombinant GyrA and GyrB were purified by Ni²⁺ affinity chromatography using modified protocol of kit manufacturer (Qiagen, Inc). In brief, the recombinant *E. coli* cells expressing these proteins were lysed in buffer A (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA and 1 mM PMSF) containing 200 μ g/ml lysozyme followed by 0.5 % Triton X-100 and 0.5 % NP40. Lysate was sonicated for 10 min at 30 s pulse with 1 min intermittent cooling at ice temperature with 50 % duty cycle. Clear cell free extract containing recombinant proteins was obtained by centrifugation at 20,000 \times g and dialyzed in buffer A containing 5 mM imidazole and passed through NiNTA agarose column (Qiagen, Inc) pre-equilibrated with buffer A. Column was washed extensively with buffer A containing 20 mM imidazole till unbound or loosely bound proteins stopped coming from the column. The recombinant proteins were eluted by applying step gradients of 100, 200, and 300 mM imidazole in buffer A. All the fractions were analysed on SDS-PAGE and the fractions containing pure protein were pooled, dialysed in buffer A and concentrated using 50 kDa cut off filters (Amicon, Inc.). Histidine tag was removed with thrombin and the mixture was further purified through ion-exchange column chromatography. Fractions showing single protein band between 150 and 200 mM NaCl on SDS-PAGE were pooled and dialysed overnight in buffer A containing 50 % glycerol and stored at -70 °C for subsequent uses.

Gel shift assay

The DNA binding activity of wild type and mutant proteins was performed by agarose gel electrophoresis as described previously (Adachi et al. 2014). In brief, 100 ng of *Eco*RI-digested pUC19 plasmid DNA was added to TA buffer (40 mM Tris–acetate, pH 8.0, 20 mM sodium acetate) containing 1 mM MgCl₂. To this 6.5 μ M or 13 μ M PprA protein was added and volume was made to 10 μ l. Assay was carried out for 10 min at 37 °C, and subjected to

0.8 % agarose gel electrophoresis using TA buffer. Gel shift assay was also employed for checking the physical interaction of purified PprA and DrGyr subunits. For that plasmid (pSK+) DNA was incubated with GyrA, GyrB, and PprA separately and in different combinations. Nucleoprotein complexes were analysed on agarose gel as described above.

Enzyme activity assay

DNA gyrase activity assays were performed as described previously (Karkare et al. 2012). In brief, purified recombinant GyrA and GyrB subunits were reconstituted in 1:2 molar ratio 15 min prior to reaction for obtaining an active DNA gyrase complex as suggested earlier (Manjunatha et al. 2002). Superhelical pBluescript SK+ plasmid DNA was gel purified from a carefully made plasmid preparation and relaxed pUC19 plasmid substrate was procured commercially (Cat. No. N0471S, New England Biolabs). For supercoiling assay, 500 ng of relaxed plasmid DNA was added to buffer containing 40 mM Tris–HCl (pH 7.9), 25 mM KCl, 1 mM DTT, 0.1 mg/ml tRNA, 100 mM potassium glutamate, 0.36 mg/ml BSA, 6 mM MgCl₂, 2 mM spermidine and 1 mM ATP. To this 150 nM reconstituted DNA gyrase was added and volume was made to 30 μ l. Assay was carried out for 60 min at 37 °C. DNA nicking assays were performed with different concentrations of reconstituted DNA gyrase in a similar way, except ATP and spermidine were omitted and the substrate was 400 ng superhelical form of pBluescript SK+ plasmid DNA prepared indigenously. Decatenation assay was done in 30 μ l volume with 300 ng kinetoplast DNA (kDNA) (TopoGEN, Inc. Florida) in 40 mM Tris–HCl (pH 7.9), 25 mM KCl, 4 mM DTT, 100 mM potassium glutamate, 0.36 mg/ml BSA, 6 mM MgCl₂ and 1 mM ATP for 60 min at 37 °C. The effects of PprA and its mutant derivatives on gyrase activity was monitored by adding the required amount of these proteins to reconstituted DrGyr and enzymatic activities were carried out as above. All the reactions were terminated with 2 μ l of 2 % sodium dodecyl sulphate (SDS), followed by proteinase K treatment for 20 min at 37 °C. Samples were extracted once with an equal volume of chloroform:isoamyl alcohol (24:1) and mixed with 0.1 volume of loading dye (50 % glycerol, 0.025 % bromophenol blue). Similarly, *E. coli* DNA gyrase was procured commercially (New England Biolabs, Inc) and all three activities like DNA relaxation, supercoiling and decatenation were measured as described above. For supercoiling assay and DNA nicking assay, products were analysed by 1 % agarose gel and ethidium bromide stained DNA bands were visualized, and quantified densitometrically as required. For decatenation assay, products were analyzed on 1 % agarose gel containing ethidium bromide. ATPase activity

of DrGyr was monitored as described earlier (Modi and Misra 2014). The Pi released was measured and enzyme activity has been represented as nM/sec.

Protein–protein interaction studies

In vivo protein–protein interaction was studied using BACTH [Bacterial Adenylate Cyclase (CyaA)-based Two Hybrid] bacterial two-hybrid system (Karimova et al. 1998) as described earlier (Kota et al. 2014b). pUT18 and pUT18C expressing T18 the C-terminal domain while pKNT25 and pKT25 expressing T25 the N terminal domain of CyaA were procured and used in this study for generating translation fusion with target proteins. T25 and T18 domains of CyaA were fused separately with target proteins and co-expressed in *cyaA*[−] strain of *E. coli* BHT101. Whenever, the two target proteins interact, both T18 and T25 domains of CyaA would have interacted too, resulting in the reconstitution of an active CyaA enzyme in *cyaA* mutant and thus the expression of β -galactosidase would be seen. In this study, the coding sequences of *pprA* mutants, *pprA397* (for K133E) and *pprA547* (for W183R), were cloned in pKNT25 vector at *Bam*HI and *Kpn*I sites yielded pKNTK133E and pKNTW183R plasmids, respectively. Similarly, the cloning of *gyrA*, *gyrB* and wild type *pprA* were carried out in respective bacterial two hybrid system plasmids as described earlier (Kota et al. 2014a). These plasmids were transformed into *E. coli* BTH-101 in different combinations. Recombinant clones harbouring both the plasmids, as monitored by co-expression of respective antibiotic resistance, were grown overnight and spotted on LB agar plate containing 200 μ M IPTG. Plate was incubated at 30 °C overnight and the appearance of blue colour colonies was recorded. In parallel, these cells were grown overnight at 30 °C in the presence of IPTG in liquid culture, and β -galactosidase activity was measured in whole cells using standard protocols (Sambrook and Russell 2001) and the specific activity of enzyme as U/mg protein was calculated as described earlier (Kota et al. 2014b).

Microscopic studies

Microscopic studies were carried out as described earlier (Charaka and Misra 2012). Both wild type and *pprA* mutant (Narumi et al. 2004) were grown overnight under normal conditions. Cells were treated with 6.5 kGy gamma radiation and allowed to recover for 2 h post irradiation. Cells were stained with Nile red (4 μ g/ml) and with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) (0.2 μ g/ml) whenever required. These cells were mounted on agarose-coated slides (1.2 %) and observed under fluorescence microscope (Axio Imager CM5, Carl Zeiss AG). Images were recorded under phase contrast, and

by excitation at 516 and 350 nm for Nile red and DAPI, respectively. Cell and nucleoid sizes were measured using Axiovision 4.8 software and statistically analysed using Graphpad Prism software.

Results

DNA Gyrase of *D. radiodurans* acts like bacterial Type II DNA topoisomerase

The recombinant GyrA and GyrB subunits of *D. radiodurans* DNA gyrase were purified (Fig. 1a) and DrGyr complex was prepared by mixing GyrA and GyrB as described in case of other bacterial gyrases (Manjunatha et al. 2002). DrGyr was checked for its plasmid DNA relaxation, supercoiling and kDNA decatenation activities. Results showed that the reconstituted enzyme is active and it could convert superhelical plasmid to relaxed form (Fig. 1b) and relaxed substrate to superhelical product under respective reaction conditions (Fig. 1c). Upon incubation of this enzyme with kDNA a highly catenated DNA substrate, it converted kDNA into its typical decatenated species that included relaxed, linear as well as superhelical products (Fig. 1d). On longer incubation, the relatively higher proportion of relaxed kDNA product was observed. Since DrGyr showed all three activities of type IIA family topoisomerase, *E. coli* gyrase was used as control for comparing these activities. Under similar conditions, *E. coli* gyrase could relax the superhelical DNA and supercoiled the relaxed DNA into corresponding products but failed to decatenate kDNA under normal physiological conditions. (Fig. S1), indicating that DrGyr is different from *E. coli* gyrase as far as decatenation of kDNA is concerned. *E. coli* has multiple DNA topoisomerases and the decatenation of intertwined circular chromosome and plasmids has been reported due to TopoIV and not gyrase under normal physiological conditions (Hiasa et al. 1994; Zechiedrich et al. 1997). *D. radiodurans* genome does not encode *E. coli* homologues of TopoIII and TopoIV (Makarova et al. 2001). These results suggested that DrGyr reconstituted from recombinant GyrA and GyrB subunits of *D. radiodurans* is an active enzyme, which seems to be a multifunctional type IIA family DNA topoisomerase in this bacterium.

DNA binding and polymerisation mutants of PprA were isolated

During the course of expression of recombinant PprA in *E. coli* (Narumi et al. 2004), it was noticed that *E. coli* BL21 (DE3) cells expressing wild type PprA grew poorly on LB agar plates. However, the cells expressing spontaneous DNA binding mutant G149E of PprA as identified from a

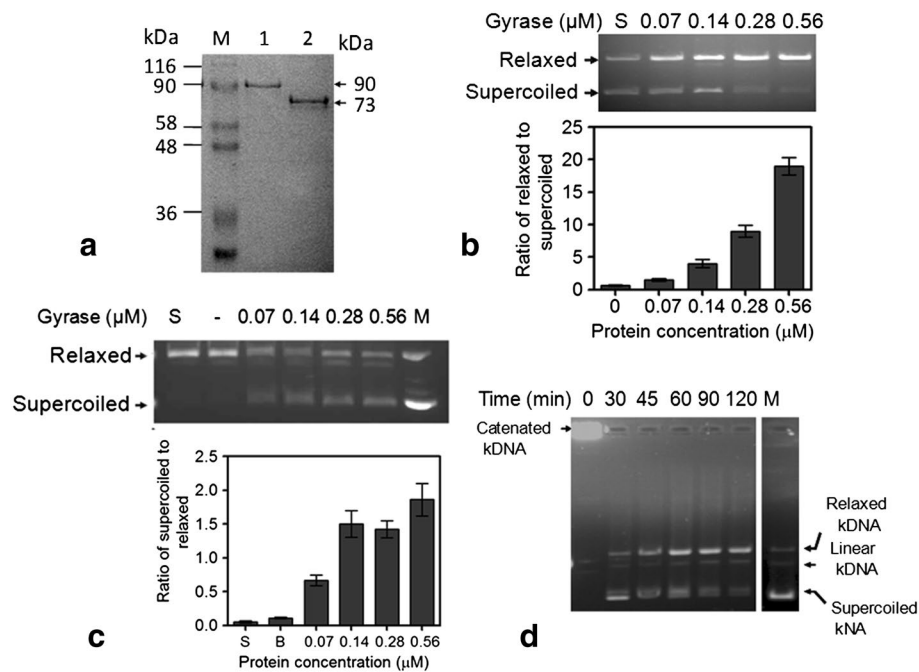


Fig. 1 Activity characterization of DrGyr. Recombinant GyrA (1) and GyrB (2) subunits of DNA gyrase of *D. radiodurans* were purified from recombinant *E. coli*. Gyrase enzyme was constituted by purified recombinant GyrA and GyrB subunits (a). For DNA relaxation assay, an increasing concentration (0.07–0.56 μM) of reconstituted DrGyr (Gyrase) was incubated with plasmid DNA (S) substrate having both relaxed (relaxed) and covalently closed circular (supercoiled) forms. Products were analysed on agarose gel (top panel), DNA bands were quantified and the ratio's of relaxed to supercoiled DNA were plotted (bottom panel) (b). For supercoiling activity assay,

the commercially procured relaxed plasmid DNA substrate (S) was incubated with assay buffer without (B) and with the increasing concentration (0.07–0.56 μM) of enzymes (Gyrase). Products were analysed on agarose gel (top panel), DNA bands intensity was quantified and the ratio's of supercoiled to relaxed DNA was plotted (bottom panel) (c). For decatenation activity assay, the kinetoplast (kDNA) DNA (S) was incubated with 0.50 μM DrGyr. Aliquots were taken at different time intervals (30, 45, 60, 90, 120 min) and the products were analysed on agarose gel (d). Data shown is from a reproducible experiment repeated two times with independent enzyme preparations

DNA repair-deficient mutant of *D. radiodurans* (Kitayama et al. 1983) grew much faster than wild type PprA expressing cells (Fig. 2a). Since, the observed slow growth was specific to when PprA was expressed on pET3a vector, but not observed when it was expressed using other pET expression vectors suggesting that a leaky expression of PprA wielded a deleterious influence on the growth of host probably by its DNA binding activity. This fact prompted us to isolate PprA mutant proteins lacking DNA binding activity by random mutagenesis and subsequently by comparing their growth characteristics with wild type PprA expressing cells. Those clones of PprA that showed comparatively faster growth were sequenced and mutation sites were mapped in *pprA* gene. As a result, several nonsense mutations and missense mutations were found in the coding sequence of *pprA*. Two missense mutations were mapped as A397G and T547A in nucleotide sequence of *pprA*, which resulted in lysine 133 changed to glutamate (K133E) and tryptophane 183 to arginine (W183R), respectively. Therefore, these two mutants K133E and W183R of PprA were used in this study and the details of other DNA binding mutant derivatives of PprA will be described elsewhere.

Recombinant PprA and its K133E and W183R derivatives were purified (Fig. 2b). Since PprA forms homopolymer even without DNA and its polymerization state is dependent on the concentrations of protein and salt (Adachi et al. 2014), the polymerization feature of PprA was checked through gel mobility shift on native polyacrylamide gel. The polymerization characteristics of K133E mutant was less affected as compared to W183R because K133E was found producing relatively larger polymers than W183R. W183R mutant migrated much faster than wild type as well as K133E in non-denaturing PAGE (Fig. 2b). Both mutants lost DNA binding activity of PprA (Fig. 2c). These results showed that K133E and W183R lost DNA binding activity of PprA completely but the polymerization characteristic was partially reduced in these single mutants.

DNA binding mutants of PprA do not affect DrGyr functions in vitro

Earlier, the interaction of PprA with GyrA was shown (Kota et al. 2014a) and here we have obtained the DNA binding mutants K133E and W183E of PprA. Therefore,

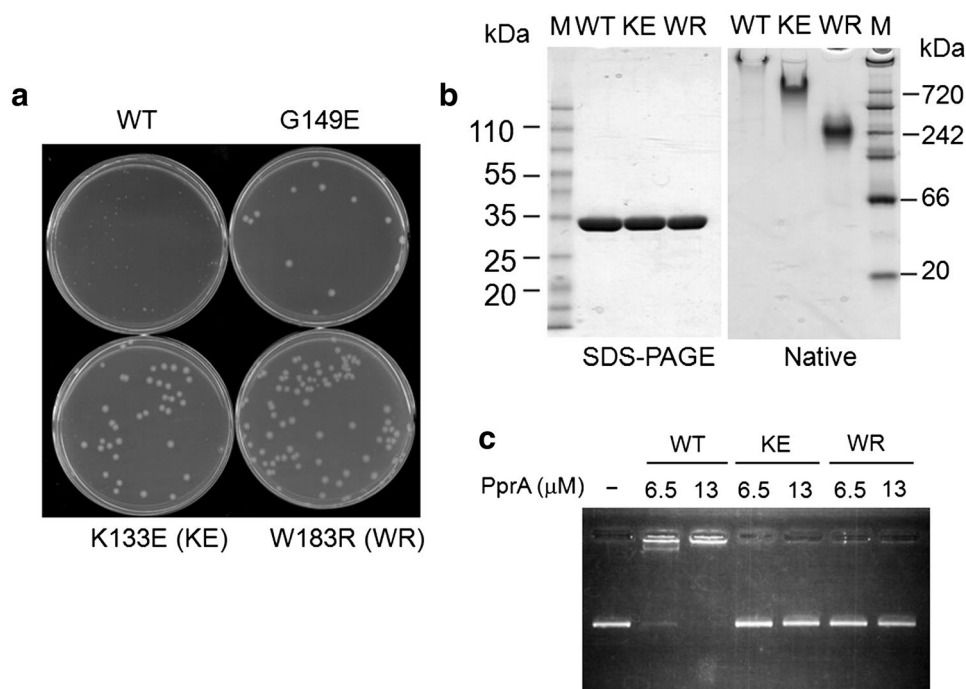


Fig. 2 DNA binding and polymerization assay of PprA and its mutants. The *pprA* gene was randomly mutagenized and the two mutants K133E and W183R were obtained as described in materials and methods. Difference in colony size of *E. coli* BL21 (DE3) producing wild type PprA (WT), and DNA binding mutants like G149E, K133E, and W183R grown on LB agar plate for 18 h at 37 °C (**a**). These proteins were expressed in *E. coli* and the wild type PprA (WT), K133E (KE) and W183R (WR) proteins were purified (SDS-

PAGE) and checked for their ability to form polymers on native PAGE (Native). Sizes of PprA monomer and polymers were compared with pre-stained protein standard and “NativeMark” unstained protein standard (Life Technologies) on SDS-PAGE and native PAGE, respectively (M) (**b**). For checking DNA binding activity, the linear dsDNA was incubated with wild type (WT), K133E (KE) and W183R (WR), and products were analysed on agarose gel (**c**)

the effects of PprA and its K133E and W183E derivatives on DrGyr activities were checked. Purified PprA at 0.4 μM concentration stimulated DNA relaxation activity of DrGyr by ~8 fold as compared to DrGyr without PprA (Fig. 3a, compare sample lane 4 with lane 6). PprA concentrations beyond 0.4 μM though continued stimulating DrGyr relaxation activity it was not proportional with PprA concentration (Fig. 3a, compare sample lane 6–10). DNA binding mutants of PprA did not show wild type effect on DNA relaxation activity of DrGyr (Fig. 3c). When decatenation function of DrGyr was measured in the presence and absence of wild type and DNA binding mutants of PprA, the decatenation of kDNA was negatively affected in the presence of PprA or its mutant derivatives (Fig. 4a) and a significant amount of catenated kDNA substrate did not get converted into their decatenated products. Further, the levels of supercoiled kDNA product from catenated kDNA were nearly same in the presence of wild type PprA and its mutants (Fig. 4b). But in the presence of wild type PprA a faster migrating band (may be supercoiled species) was observed on gel which was absent in the presence PprA mutant derivatives and with DrGr alone (Fig. 4a).

However, when the effect of PprA on supercoiling activity of DrGyr activity was measured on the relaxed plasmid substrate, only wild type PprA inhibited this activity and not its K133E and W183R mutants (Fig. 4c). PprA alone did not metabolize kDNA. Since PprA is a DNA binding protein, the possibility of its DNA binding activity *per se* if responsible for DrGyr activity modulation was argued and DrGyr activity on kDNA was examined in the presence of DrRecA, which neither metabolized catenated kDNA itself nor influenced decatenation functions of DrGyr. These results suggested that PprA differentially regulates DrGyr activities in vitro.

W183R mutant of PprA forming short oligomers does not interact with GyrA

To understand whether PprA’s influence on DrGyr activities requires the physical interactions of these proteins, the wild type PprA and its K133E and W183R derivatives were checked for in vivo interaction with both GyrA and GyrB subunits of DrGyr, using bacterial two hybrid system. Despite loss of DNA binding activity in K133E mutant, the level of its interaction with GyrA subunit was

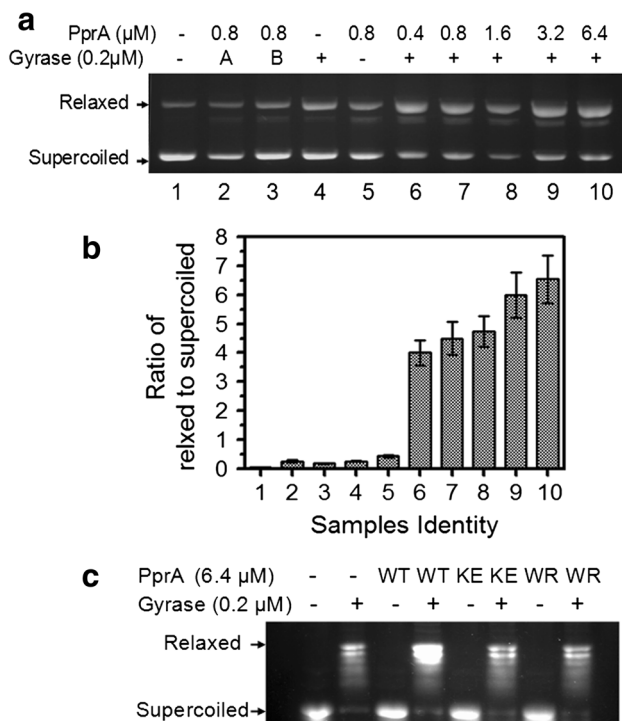


Fig. 3 Effect of PprA on DrGyr activity. 400 ng of pBlue-scriptSK + plasmid DNA containing majority supercoiled form was incubated with $0.2\mu\text{M}$ gyrase A (A), $0.2\mu\text{M}$ gyrase B (B) subunits and $0.2\mu\text{M}$ DrGyr complex in the presence and absence of different concentration of wild type PprA. Products were analysed on normal agarose gel (a). The intensity of DNA bands representing the relaxed and supercoiled species were quantified densitometrically and the ratios of relaxed to supercoiled species were plotted (b). Similarly, the supercoiled plasmid DNA was purified and incubated with $0.2\mu\text{M}$ DrGyr (Gyrase) with and without wild type (WT) and K133E (KE) and W183R (WR) mutant derivatives. Products were analysed on agarose gel (c). Data shown are representative of reproducible experiments repeated at least three times

almost same as that of wild type PprA (Fig. 5). However, W183R mutant failed to interact with GyrA *in vivo*. None of the PprA derivatives could establish physical interaction with GyrB subunit (Fig. 5b). However, GyrA and GyrB subunits interacted each other confirming the correctness of these constructs and functional interaction of these proteins *in vivo*. Physical interaction of GyrA with GyrB and GyrA with PprA were demonstrated earlier (Kota et al. 2014b). In other bacteria, GyrB is known to contribute to DrGyr functions through its ATPase activity therefore; PprA effect on ATPase activity of GyrB as well as DrGyr enzyme was monitored. There was no effect of PprA or DNA on ATPase activity of either GyrB or DrGyr (Fig. 6). These results indicated that DNA binding activity of PprA is not required for its interaction with DrGyr but PprA's polymerizing characteristics is important. Further, PprA inhibition of DNA supercoiling does not seem to be through the inhibition of ATPase

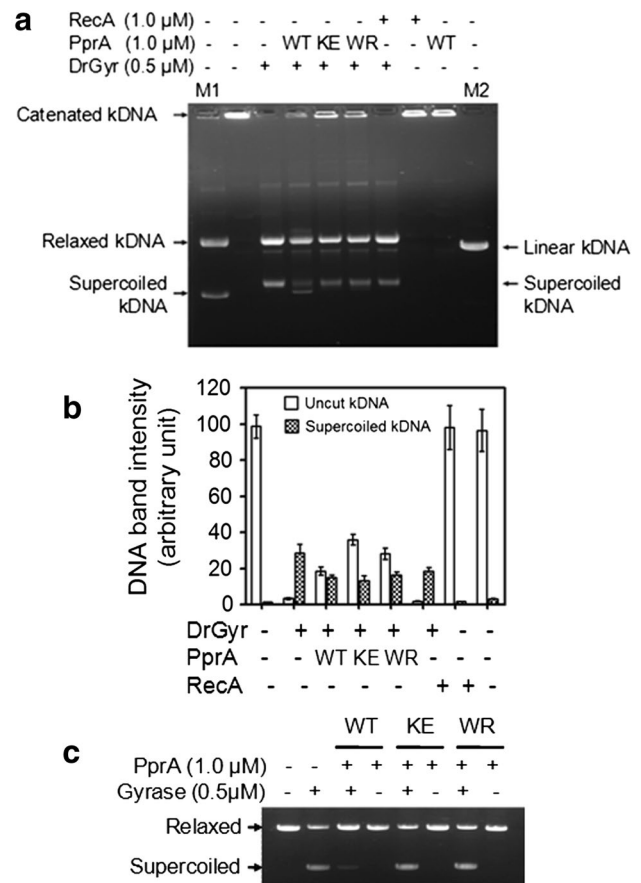


Fig. 4 Effect of PprA and its mutants on decatenation and supercoiling activities of DrGyr. Decatenation function of DrGyr (DrGyr) was checked with kDNA substrate incubated with $0.5\mu\text{M}$ enzyme (DrGyr), wild type PprA (WT) and its K133E (KE) and W183R (WR) and recombinant deinococcal RecA as negative control, in different combinations as shown in figure (a). Reaction products were analysed on agarose gel and decatenated species were characterized by comparing their mobility's with nicked and relaxed kDNA monomers in marker (M1) and linear kDNA monomer marker (M2). The amount of catenated kDNA substrate present in the wells as well as the supercoiled species in respective samples were measured densitometrically and plotted in graph (b). Similarly, DrGyr (Gyrase) activity was checked on relaxed plasmid DNA incubated with $1.0\mu\text{M}$ PprA and its derivatives (c). Data shown here are the representatives of respective reproducible experiments repeated at least two times independently

activity of GyrB, rather to be through DNA–DrGyr/PprA interactions.

PprA mutant showed bigger nucleoid and cell size

PprA's role in cell division and genome maintenance in *D. radiodurans* in response to γ radiation treatment has been shown recently (Devigne et al. 2013; Kota et al. 2014b). Therefore, the effect of PprA absence in *pprA* mutant on the size of the *D. radiodurans*'s cell and its DAPI stained nucleoid was examined microscopically in

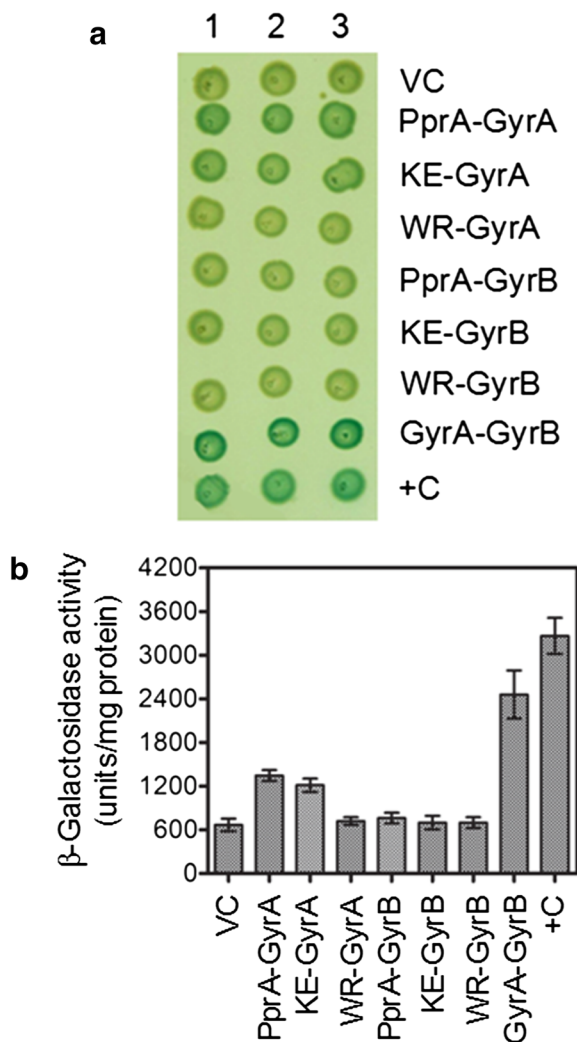


Fig. 5 PprA interaction with DNA gyrase subunits of *D. radiodurans*. The gyrase A was cloned in pUT18 (pUTgyrA) and gyrase B in pKNT25 (pKNTgyrB) vectors of bacterial two hybrid system and the recombinant fusion proteins were expressed as detailed in materials and methods section. The wild type PprA (PprA) was cloned in both pKNT25 (pKNTpprA) and pUT18 (pUTpprA) plasmids. Similarly, K133E (KE) and W183R (WR) were cloned in both the vectors yielded pKNT397 and pUT397, and pKNT547 and pUT547, respectively. These plasmids were co-transformed in different combinations into *E. coli* BTH101. Care was taken that each combination should have both the domains of CyaA (T18 and T25) expressing along with two different proteins. *E. coli* BTH101 was also co-transformed with pUT18 and pKNT25 (VC), and pKNTcheA and pUTcheA (+C) and used as respective controls. Three independent clones (1, 2, 3) of these samples were spotted on X-gal plate (**a**) and also monitored for the expression of β -galactosidase in liquid culture (**b**). β -Galactosidase activity above 700 units/mg protein was considered as positive for physical interaction of two proteins. Data shown in panel 'b' are the mean \pm SD ($n = 4$) and in c is the representative of a reproducible experiment repeated 2 times independently

response to DNA damage. The *pprA:cat* mutant showed larger cell size ($2.6430 \pm 0.0389 \mu$) as compared to wild type ($2.0135 \pm 0.0419 \mu$) (Fig. 7). Interestingly, the size

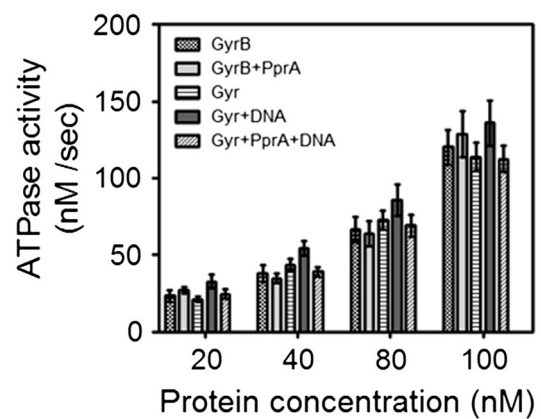


Fig. 6 Effect of PprA on ATPase activity of DrGyr in vitro. Different concentrations (20, 40, 80 and 100 nM) of recombinant gyrase B subunit (GyrB), and DrGyr (Gyr) reconstituted from gyrase A and gyrase B subunits were incubated with 1 μ M PprA and 100 ng plasmid DNA for 1 h at 37 $^{\circ}$ C. Release of inorganic phosphate (Pi) was estimated as described in methods and converted into nM/s. Data shown are the mean \pm SD ($n = 3$)

of DAPI stained nucleoid in *pprA:cat* ($1.9564 \pm 0.0324 \mu$) was also more than the size of nucleoids in wild type ($1.6437 \pm 0.0345 \mu$) cells. The molecular basis of cell and nucleoid's size increase in the absence of PprA is not known yet. However, the possibility of some defect in proper segregation of duplicated genome may led to an increase in DNA content and affected cell division, which led to an increase in cell volume cannot be ruled out. These results suggest that PprA plays important role in genome maintenance in this bacterium.

Discussion

The extraordinary radioresistance in *D. radiodurans* is largely attributed to its efficient DSB repair and the protection of biomolecules from oxidative damage (Zahradka et al. 2006; Krisko and Radman 2013). This bacterium also harbours a multipartite genome system comprising of two chromosomes and two plasmids. These genome elements are present in 4–10 copies per cell (Minton 1994) and packaged together and form toroidal structure of the nucleoid. The *D. radiodurans*'s nucleoid proteome analysis showed the presence of both the subunits of DNA gyrase but not PprA (de la Tour et al. 2013). Same study reported the presence of PprA in the nucleoid of *Deinococcus deserti*. PprA homologues have been found in the genome of all members of *Deinococcaceae* family, sequenced till date. This indicated the importance of PprA in the radioresistance in these bacteria. Although nucleoid proteome analysis has ruled out PprA presence in nucleoid, the recruitment of PprA in *D. radiodurans*'s nucleoid has been demonstrated

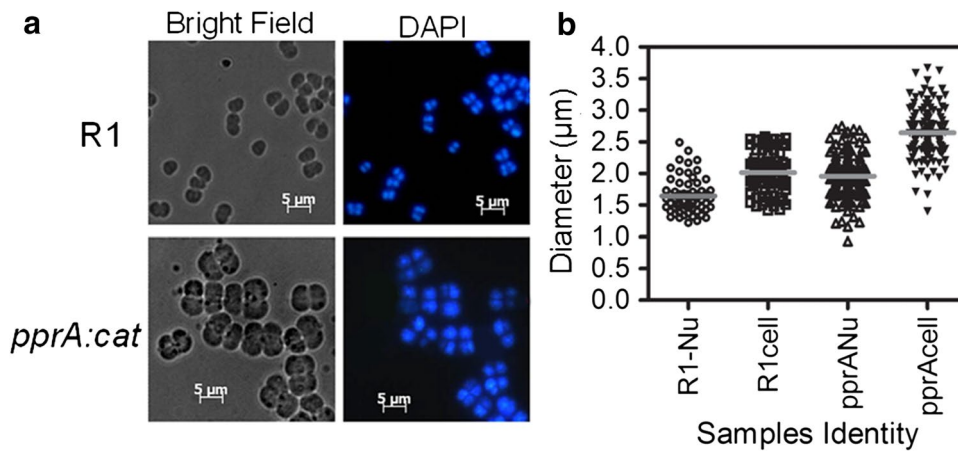


Fig. 7 Effect of *pprA* mutation on nucleoid and cell size in *D. radiodurans* exposed to gamma radiation. Both wild type (R1) and *pprA* mutant (*pprA:cat*) cells were grown, stained with DAPI and imaged under phase contrast for cells (Cells) and under DAPI fluorescence

for nucleoid content (a). Sizes of DAPI stained nucleoid (R1-Nu and PprANu) and cells (R1cell and pprAcell) were measured in nearly 300–500 cells for each (b). Data was analysed in Graphpad prism for standard error ($n > 300$)

independently (Devigne et al. 2013; Kota et al. 2014b). These findings indicated that PprA does not seem to be a permanent resident of nucleoid but the function dependent interaction of PprA with the nucleoid cannot be ruled out. Further, the possibility of PprA interacting with nucleoid through some other proteins having roles in genome maintenance and γ radiation resistance could be argued. Recently the physical interactions of PprA with GyrA and TopoIB of *D. radiodurans* have been demonstrated (Kota et al. 2014b). It was observed that when copy number of *gyrA* gene was reduced to a certain level in both wild type and *pprA* mutant of *D. radiodurans*, these cells debilitated under normal conditions, and there was no further loss in γ radiation resistance of *pprA* mutant (Kota et al. 2014a). This extends the paradigm on indispensability of bacterial GyrA also in case of *D. radiodurans*.

Here, we have characterized DNA gyrase from *D. radiodurans* and demonstrated that enzyme constituted from recombinant GyrA and GyrB subunits exhibits all the important activities of type IIA bacterial DNA topoisomerases like DNA relaxation measured as nicking, supercoiling and decatenation in vitro. Topoisomerases are the ubiquitous genome maintenance enzymes in bacteria. In *E. coli*, the decatenation of intertwined circular chromosome or catenated plasmid is resolved by DNA topoisomerase III and IV. *E. coli* gyrase does not catalyze decatenation of chromosome or plasmid at physiological salt concentration (Hiasa et al. 1994). Similar observation was recorded here also with *E. coli* DNA gyrase (Fig S1). Decatenation of duplicated intertwined circular chromosome is shown to be a pre-requisite for faithful inheritance of genetic materials and cell division in *E. coli* (Espeli et al. 2003). Since PprA showed in vivo interaction with GyrA and TopoIB (Kota

et al. 2014a, b), the impact of these interactions in nucleoid remodelling could be suggested. Recently PprA involvements in cell division and genome segregation have been reported (Devigne et al. 2013). Further, we observed that both cell and nucleoid in *pprA:cat* mutant were larger in size than wild type cells during DNA damage recovery process (Fig. 7). This also supported the possible role of PprA in nucleoid packaging, perhaps by interacting with other nucleoid proteins. The stimulation of T4 DNA ligase, *E. coli* Ligase and *Deinococcus* LigB activity by PprA had already been shown earlier (Narumi et al. 2004; Kota et al. 2010). PprA is found to be the partner of LigB and TopoIB in multiprotein complex characterized from *D. radiodurans* (Kota and Misra 2008). Based on these findings, it is reasonable to speculate that PprA seems to be a ubiquitous post translational regulator of DNA repair and genome maintenance proteins in *D. radiodurans*. In this direction, this study has brought forth some evidence to suggest that PprA plays an important role in differential regulation of DrGyr activities at least in vitro. Since PprA is known to preferentially bind to the open ends of double-stranded (ds) DNA in vitro (Narumi et al. 2004) and recognize DNA breaks in vivo (Satoh et al. 2006), the possibility of PprA inhibiting only those steps in DrGyr catalytic mechanism that involves reutilization of dsDNA ends could be speculated. Our results that wild type PprA could stimulate DNA nicking but inhibited supercoiling activity which was not observed with DNA binding mutants (K133E and W183R) supported the speculation. Since PprA homologues are found in many species of *Deinococcus*, the amino acid sequence alignment was carried out to check if K133 and W183 are conserved across *Deinococci*. Interestingly, we found that the amino acid corresponding to W183 in *D. radiodurans* was conserved in all

the species while four of them have lysine corresponding to K133 and others have arginine in place of K133 in *D. radiodurans* (Fig. S2). This indicated the possible involvement of K133 and W183 of PprA in radioresistance perhaps by modulating DrGyr functions. The stimulation of nicking and decatenating activities of TopoIV by SeqA, a protein associated with the regulation of replication initiation has been reported in *E. coli* (Kang et al. 2003). DNA gyrase activity inhibition by DNA binding proteins as well as those containing pentapeptide repeats and thus potentially mimicking the helical structure of dsDNA has also been reported in other bacteria (Shah and Heddle 2014). For example, GyrI from *E. coli* and MfpA from *Mycobacterium* sp. inhibit respective DNA gyrases by interfering with gyrase interaction with DNA substrates (Nakanishi et al. 1998; Chatterji and Nagaraja 2001; Montero et al. 2001). Similarly, MufI from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* inhibits gyrase activity from these bacteria (Sengupta et al. 2006; Sengupta and Nagaraja 2008).

In conclusion, we showed that enzyme constituted from recombinant GyrA and GyrB subunits of *D. radiodurans* was active showing all three important functions of type IIA bacterial DNA topoisomerases. PprA interacts with GyrA subunit most likely through its polymerization domain while PprA attenuation of DNA supercoiling activity is through its DNA binding activity. Future studies can test independently, whether PprA interaction with DrGyr has physiological relevance like (1) in maintaining the required topology of the *Deinococcus* genome, (2) in the expression of genes required for stress tolerance and (3) in the duplication and faithful segregation of genome during normal cell division. The significance of PprA recruitment to nucleoid that had DrGyr subunits during post DNA damage recovery would be another interesting aspects worth understanding. From the available results, however, it is important to emphasize that the *D. radiodurans* genome encodes active DNA gyrase exhibiting all three activities of bacterial type II topoisomerase, which are catalyzed by multiple enzymes in other bacteria, and PprA differentially regulates these functions.

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