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Research Paper

ParA Encoded on Chromosome I of *Deinococcus radiodurans* Requires its Cognate ParB and Centromere for its Dynamics

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The presence of multiple sets of ParA and ParB genome partitioning proteins in bacteria harboring multipartite genomes raises doubt about their functional redundancy. *Deinococcus radiodurans* is a highly stress tolerant bacterium that harbors multipartite genome system comprising chromosome I, chromosome II and plasmids. ParAs encoded on these elements were found to be different in terms of their primary sequences and eventually clustered in the separate phylogenetic groups. As known earlier, ParB1 (ParB encoded on chromosome I) showed sequence specific interaction with its cognate *cis* element (centromere). ParA of chromosome I (ParA1) produced larger size complex in the presence of dsDNA and ATP, which was reduced to smaller size upon incubation with ParB1- centromere complex *in vitro*. GFP-ParA1 expressing in recombinant *Escherichia coli* showed a dynamic change in its cellular localization during cell division only when both ParB1 and centromere co-existed with ParA1. These results suggested that ParA1 requires ParB1 and its cognate centromere for its cellular dynamics, which would eventually drive the separation of duplicated daughter chromosomes to opposite poles during cell division.

Key Words: A-loop ATPase; *Deinococcus*; Multipartite Genome; ParA1 Partitioning Protein; Radioresistance

Introduction

Deinococcus radiodurans is characterized for its extreme resistance to DNA damaging agents including radiations and desiccation (Battista, 2002; Slade and Radman, 2011). A highly efficient DNA double strand break (DSB) repair and a strong oxidative stress tolerance are amongst the mechanisms that are attributed to the extreme phenotypes of this bacterium (Blasius *et al.*, 2008; Misra *et al.*, 2013). Unlike majority of the prokaryotes, *D. radiodurans* harbors multipartite genome system comprised of chromosome I (~2.65Mb), chromosome II (~0.412Mb), megaplasmid (~177kb) and small plasmid (~46kb) and the ploidy (White *et al.*, 1999). Genome of this bacterium is highly compact and all the genome elements exist together in the form of toroidal structure (Levin-Zaidman *et al.*, 2003). Genome sequence analysis shows that chromosome I contains the genes encoding the majority of the functions associated with normal growth of the bacterium while chromosome II and megaplasmid encode proteins that seems to be contributing to its extraordinary radioresistance and DNA damage response (Makarova *et al.*, 2001). Some of these include the stress response regulators, two component systems, serine/threonine protein kinases and some DNA repair proteins e.g. PprA, which is annotated only in the genomes of *Deinococcaceae*. Therefore, the mechanisms contributing to genome complexity and its maintenance in *D. radiodurans* would be worth studying.

Mechanisms underlying genome segregation are better understood in bacteria maintaining single circular chromosome and low copy number plasmids (Ghosh *et al.*, 2006; Gerdes *et al.*, 2010). Till recently,

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a very little is known about the maintenance of multipartite genome in bacteria. Now we know that there are many bacteria that harbor multipartite genome system including *Vibrio cholerae* (Heidelberg *et al.,* 2000), *Burkholderia cenocepacia* (Dubarry *et al.,* 2006), *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* (Kahng and Shapiro, 2003) and *D. radiodurans* (White *et al.,* 1999). Recently, it has been reported that both the chromosomes of *Vibrio cholerae* encode independent replication and segregation machineries (Egan and Waldor, 2003; Fogel and Waldor, 2003). Further, unlike *D. radiodurans* where all the copies of multipartite genome elements are packaged in toroidal form, both the chromosomes of *V. cholerae* are spatially separated in the opposite poles in the cells. Multipartite genome of *D. radiodurans* are annotated with four *parAB* operons, one each in chromosome I and chromosome II, and two operons in megaplasmid. These encode putative ParAs and ParBs, which show homology with the genome partitioning proteins characterized in other bacteria (Bignell and Thomas, 2001; Hayes and Barilla, 2006) and therefore, offering the possibility of a fine regulation of their functions in segregation of respective cognate genome elements. Mechanistically, ParB binds with *cis* element(s) (*hereafter* referred as centromere(s) on genome and act as a central point to genome segregation and eventually in the separation of duplicated genome copies to opposite poles in the cells (Gerdes *et al.,* 2010; Pratto *et al.,* 2008; Lin and Grossman, 1998; Bouet *et al.,* 2007). The Par-family ATPases fall into two distinct phylogenetic groups; Type I ParAs contain the conserved Walker-box ATP-binding motif, whereas Type II ParAs are structurally related to eukaryotic actin. Although, both Type I and Type II ParAs form ATP-dependent filamentous polymers *in vitro,* the mechanism of action is found to be different (Gerdes *et al,* 2010; Bouet *et al.,* 2007; Castaing *et al.,* 2008; Lee and Grossman, 2006; Hester and Lutkenhaus, 2007; Leonard *et al.,* 2005). Type II ParA's appear to mediate plasmid segregation by polymerizing between plasmid pairs in presence of ATP and "pushing" them apart toward the poles (Gerdes *et al.,* 2010). While the mechanism by which Type I ParAs function is less clear, this class of ParAs polymerizes through chromosome and oscillates throughout the cells. Genome segregation and cell division are interdependently regulated processes in bacteria (Wu and Errington, 2004). Identity of centromere(s) and functions of putative ParAs and ParBs were not known in *D. radiodurans*. We identified for the first time, multiple sequence motifs having centromere function and named them as *segS1, segS2* and *segS3*, in the chromosome I of *D. radiodurans.* Further, we demonstrated that ParA1 encoded on chromosome I functions like Type Ia as reported in other bacteria (Charaka and Misra, 2012). Furthermore, it is shown that ParA of chromosome II (ParA2) in the absence of its ParB (ParB of chromosome II) plays a role in regulation of cell division (Charaka *et al.,* 2013). Here we report the biochemical characterization of recombinant ParA1 and demonstrated that ParA1 undergoes polymerization /depolymerization dynamics in the presence of ParB1 and its cognate centromere *in vitro,* and its *in vivo* cellular dynamics also require ParB1 and cognate centromere.

Materials and Methods

Bacterial Strains and Materials

D. radiodurans R1 strain ATCC13939 is a generous gift from Prof. J. Ortner, Germany (Schaefer *et al.,* 2001). *E. coli* expression vector pET28a-(+) (Novagen Inc), pDSW209 (Weiss *et al.,* 1999) and p11559 (Lecointe *et al.,* 2004) and its derivative pVHS559 (Charaka and Misra, 2012) were obtained from respective sources. *D. radiodurans* is maintained in TGY (0.5 % Bacto Tryptone, 0.3 % Bacto Yeast Extract, 0.1 % Glucose) broth or on agar plate as required at 32ºC while *E. coli* derivatives harboring different plasmids are maintained in LB medium supplemented with appropriate antibiotics. All the molecular biology grade chemicals including restriction enzymes and DNA modifying enzymes were purchased from Sigma Chemical Company, USA, Roche Biochemicals, Germany, New England Biolabs, USA and Bangalore Genie, India. Recombinant techniques employed in this study were as described in (Sambrook and Russell, 2001).

Bioinformatic Analysis

The functional motif search and structure prediction studies were carried out using standard on-line bioinformatics tools and as described earlier (Das and Misra, 2011). In brief, the amino acid sequences of DR_0013 (hereafter designated as ParA1), was subjected to a PSI-BLAST search with SWISS-PROT database with "genome-partitioning proteins" as key words. The sequences obtained were aligned by CLUSTAL-X, for functional motifs search. The sequences of close homology were aligned by T-COFFEE and the conserved motifs were marked. Phylogenetic tree was constructed using these sequences. Secondary structure was inferred from PSIPRED, JNET and Prof at the Quick2D server at Max-Planck Institute for Developmental Biology. The 3D models were generated on-line as described earlier (Das and Misra, 2013).

Construction of Expression Plasmids

Genomic DNA of *D. radiodurans* R1 was prepared as published previously (Battista *et al.*, 2001) and DR_0012 (ParB1) and DR_0013 (ParA1) ORFs were PCR amplified from genomic DNA using dr0012F (5' GGAATTCCATATGGTGTCGAAAAAA TCTAGCCT 3' and dr0012R (5'CCGCTCGAG TTATTCCTCGGCCTCGTA 3') primers for *parB1* and dr0013F (5'GGAATTCCATATGATG ACGGACCACGCGGGC3) and dr0013R (5'CCGCTCGAGCTAGATTTTTTCGACACGTTGCA3') primers for *parA1* and pET0012 and pET0013 plasmids expressing ParA1 and ParB1, respectively were constructed as described earlier (Charaka and Misra, 2012). For making GFP translation fusion, *dr0012-dr0013* operon was PCR amplified using forward primer OpF (5' CGGGATCCATGA CGGACCACGCGGGC 3') and reverse primer OpR (5' CCCAAGCTT TTATTCCTCGGCCTCGTA 3') having *Bam*HI and *Hind*III sites incorporated at the 5' of the primers. PCR product was cloned at compatible sites in pDSW209 (Clontech Laboratories, Inc) to yield pDSWCIO (Charaka, 2013). The pDSWCIO was transformed into *E. coli* MG1655 for the expression and fluorescence microscopic studies as described earlier (Charaka and Misra, 2012). For expressing GFP-ParA1 fusion into *D. radiodurans*, the *gfp-parA1* chimeric DNA fragment was PCR amplified using VHSF (5 CGCGAGCTCATGAGTAAAGGAGAAGAACTTTTCA 3) and VHSR (5' CCGCTCGAGCTAGA TTTTTTCGACACGTTGCA3') primers. PCR product was cloned at *Sac*I and *Xho*I sites in pVHS559 and pVGFPA1 obtained. The centromere (*segS3*) in chromosome I of *D. radiodurans* was cloned in pDAG203 (Lemonnier *et al.*, 2000) to produce pDAGS3 as described earlier (Charaka and Misra, 2012). The *par*B1 deletion mutant of *D. radiodurans* was generated using strategy as described earlier (Khairnar *et al.*, 2008). In brief the pNOKparB1 was constructed as described by Charaka and Misra (2012) and transformed into *D. radiodurans*. Transformants were grown under selection pressure and homogeneous replacement of *par*B1 with *npt*II was confirmed by PCR amplification.

Expression and Purification of Recombinant Protein

Recombinant plasmids were transformed into *E. coli* BL21 DE3 pLysS and the recombinant proteins were purified by nickel - affinity chromatography as mentioned in Kota *et al.* (2010). The purified proteins were refolded by serial dilution of urea with concurrent increase in dithiothreitol (DTT) concentration and re-purified under native conditions using nickel affinity column buffer supplemented with 10% glycerol and 2% ethanol. Finally the fractions showing pure protein were pooled and dialyzed in a buffer (20mM Tris-HCl, 50 mM NaCl, 1mM DTT, 1mM EDTA, 1 mM PMSF and 50% glycerol) and stored in small aliquots at -20ºC till further use.

DNA-Protein Interaction Studies

For DNA binding activity assay, the 284bp DNA fragment containing *segS3* was PCR amplified from genomic DNA from *D. radiodurans* as described earlier (Charaka and Misra, 2012). In parallel, the 200bp nonspecific DNA substrates named, as DN1 and DN2, were PCR amplified using DN1F (5'

CTACACTGAGACGTTCT 3') and DN1R (5' CACGTTGATGGCGAGCA 3') for DN1 and DN2F (5' AGGCTGATCTCATTGCCA 3') and DN2R (5' TCGCCAATCTCTTGCTGA 3') for DN2. The DNA was purified from gel (QIAgen Inc., Germany) and labeled at 3' end with dig-dUTP (Roche Biochemicals, Germany) following manufacturers protocols. 0.5µM labeled substrate was incubated with increasing concentration of protein (100-1250ng) in a 20µl reaction mixture in DNA binding buffer (50mM HEPES, pH8.0, 100µM NaCl, 5mM $MgCl_2$, 30mM Na-Acetate) at 37ºC for 20 min. For ParB1 interaction with *segS3*, 0.5µM of labeled DNA substrate was incubated with increasing concentration of ParB1 as shown in figure legends. For competition experiments, 0.5µM labeled probe was pre-incubated with 500ng of ParB1 and then chased with increasing concentration of either *segS3* (for specific competition) or DN1 (for non-specific competition) as described above and also indicated in respective figure legends. Reaction mixtures were separated on 5% native PAGE and Digoxigenin labeled DNA probe was immunoblotted with anti-digoxigenin-AP antibodies (Roche Biochemicals, Germany) and signals were detected using NBT/BCIP (Roche Biochemicals) color reagent using manufacturer's protocol.

Sedimentation Analysis

Approximately 1µg of purified recombinant ParA1 was incubated with ~100ng of recombinant ParB1 and 3nM *segS3* in reaction buffer containing 50mM HEPES pH 8.0, 100mM NaCl, 5mM $MgCl_2$, 30mM Na-acetate in the presence and absence of different nucleotides at room temperature for 20 min. Mixture was centrifuged at 22000 × g for 30 min. Supernatants and pellets were separated and analysed on 10% SDS-PAGE, and stained with coomassie brilliant blue. Density of protein band was quantified densitometrically.

ATP Binding and ATPase Activity Assay

Approximately 500ng ParA1 and 200ng ParB1 were incubated with 5µci [^{32}P] α ATP and non-specific DNA (DN1) in different combinations. Mixture was incubated at room temperature for 10 min and chilled on ice before exposure to UV (300nm) as described earlier (Misra *et al.*, 1998). The mixtures were separated on SDS-PAGE, gel was dried and autoradiogram was developed. ATPase activity of recombinant ParA1 was checked using a modified protocol as described in (Kota *et al.*, 2010). In brief, 100ng of purified ParB1 was incubated with increasing molar ratio of ParA1 (1:1 to 1:10) in the presence and absence of both *segS3* and non-specific dsDNA in 50µl reaction mixture containing 50mM HEPES pH 8.0, 100mM NaCl, 5mM $MgCl_2$, 30mM Na-acetate, for 20 min at 37ºC. Reaction was stopped using malachite green reagent. The release of Pi from ATP was measured at 630nm and levels of Pi quantified using standard procedure essentially described in (Geladopoulos *et al.*, 1999). ATPase activity was calculated as nmoles Pi formed / min/ mg protein.

Expression of GFP-ParA1 and Fluorescence Microscopic Studies

E. coli cells harboring pDSWCIO were grown in LB supplemented with ampicillin (100µg /ml) and induced with 200µM IPTG using standard protocol. Similarly, *D. radiodurans* cells were transformed with pVGFPA1 plasmid and cells were induced with 10mM IPTG for 20h at 32ºC. Expression of recombinant proteins was confirmed by immunoblotting with GFP antibodies using protocols described earlier (Misra *et al.*, 2006). Fluorescence microscopy of the cells expressing GFP-ParA1 was carried out as described in Charaka *et al.* (2013) using Zeiss AxioImager (Carl Zeiss) equipped with Zeiss AxioCam MRm camera. In brief, 5 µl cells expressing these proteins in different combinations were mounted onto 1% agarose coated slide and time lapsed fluorescence microscopy was carried out at different interval at 460nm excitation and emission peak at 509nm, on Axio Imager M1 Florescence Microscope (Carl Zeiss). Images were processed using Adobe Photoshop CS3 and Image J softwares.

Results and Discussion

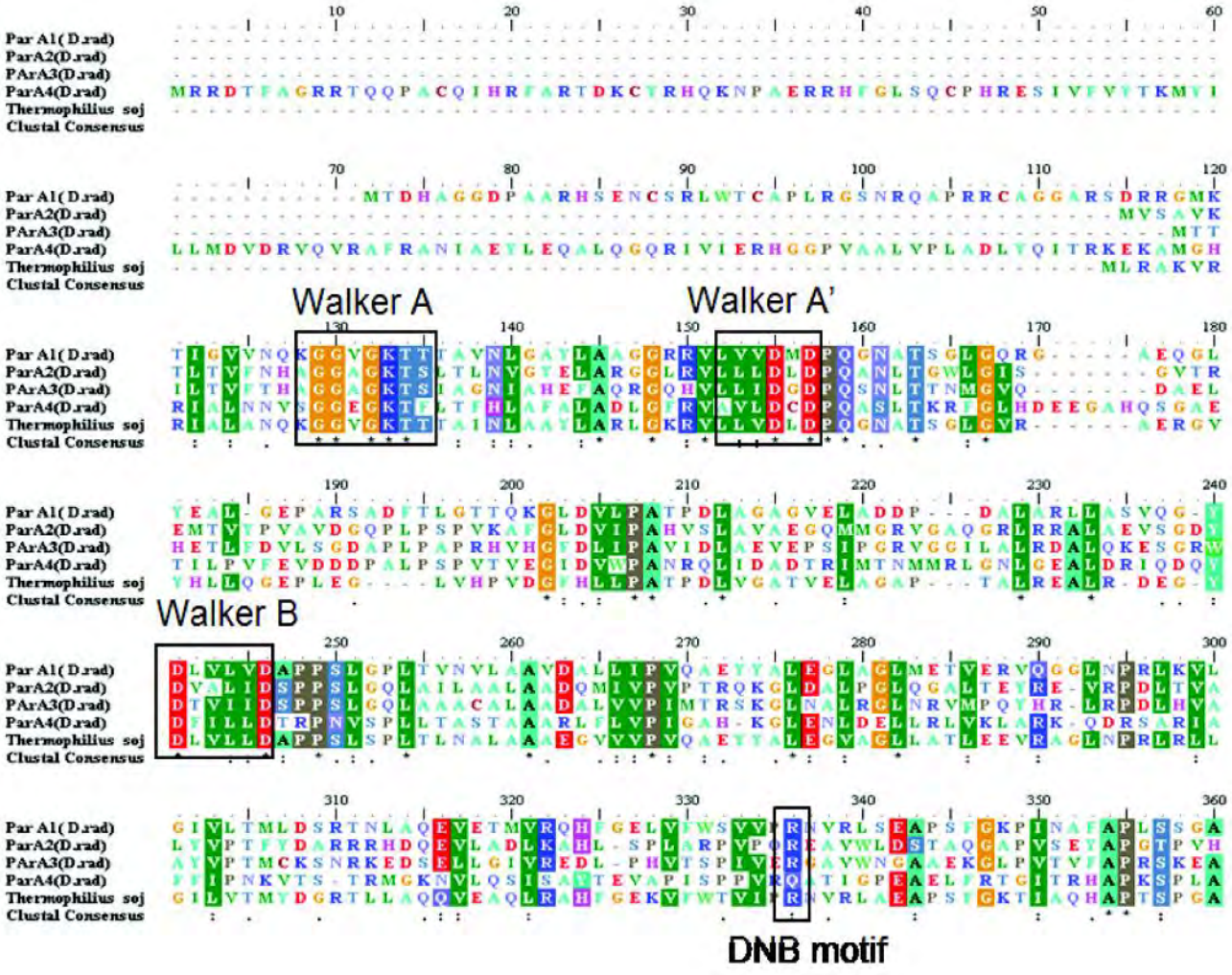
ParA1 is Closer to Chromosomal Type and Different from Other Deinococcal ParAs

Deinococcus radiodurans genome encodes four putative ParA proteins, one each on chromosome I (ParA1) and chromosome II (ParA2), and two on megaplasmid (ParA3 and ParA4). Amino acid sequences of these ParAs were compared for homology amongst each other and with ParA homologues in other bacteria. Interestingly, it was observed that all four ParAs are different from each other at amino acid levels (Fig. 1A). ParA2, ParA3 and ParA4 showed 22-33 % identity with ParA1 while levels of identities amongst ParA2, ParA3 and ParA4 were in the range of 28-42%. In spite of that, all of these have conserved functional domains like Walker A, Walker A' and Walker B and DNA binding motifs similar to that characterized in ParAs or ParA like proteins in other bacteria. As we know that proteins containing well defined Walker domains bind to nucleotide triphosphate (NTP) and hydrolysis of NTPs, if occurs, is differentially regulated (Walker *et al.*, 1982). Also it has been shown that DNA binding motif having conserved arginine residue in ParA, is essential for its role in genome segregation (Sambrook and Russell, 2001). ParA1 is closer to chromosomal ParAs characterized from other bacteria, while other deinococcal ParAs clustered distinctly in the different phylogenetic groups (Fig. 1B). ParA1 and ParA4 have N-terminal extension, which were not observed in other ParAs. The functional significance of N-terminal extension, which seems to be an apparent feature in several deinococcal proteins, is due for investigation independently. The Soj protein, one of the best characterized chromosomal partitioning proteins, from *Thermus thermophilus*, shows maximum similarity with ParA1. The 3D modeled structure of ParA1 generated based on the X-ray crystal structure attributes of Soj protein showed considerable overlaps (Fig. 1C) in functional domain, except certain regions, which formed different secondary structures in ParA1 and were missing in Soj protein. Except these small differences ParA1 seems to be closer to chromosomal ParAs characterized from bacteria and has nearly conserved functional domains. Therefore, the functional significance of ParA1 interaction with ATP, regulation of its ATPase activity and DNA binding activity in genome segregation were studied further.

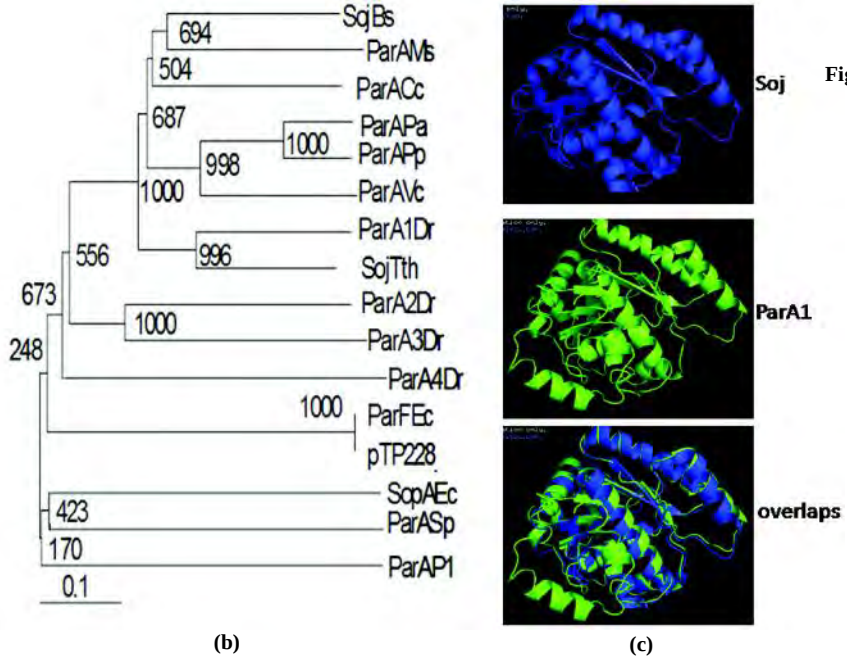
Recombinant ParA1 Binds to ATP and dsDNA

Both recombinant ParA1 and ParB of chromosome I (ParB1) were purified from recombinant *E. coli* expressing these proteins (Fig. 2). Unlike ParB1, ParA1 showed ATP binding activity, which did not change in the presence of either ParB1 or ParB1 co-incubated with DNA indicating that ATP binding to ParA1 *per se* does not get affected by either ParB1 or dsDNA (Fig. 3A). Different amount of purified ParA1 was incubated with 3 different types of dsDNA substrates like DN1, DN2 and *segS3* (Charaka and Misra, 2012) and the binding constants of ParA1were determined. We observed that ParA1 could interact with all the dsDNA substrates at almost similar affinity. The Kd values for DN1, DN2 and *seg*S3 were 351.93 ± 52.65, 267.73 ± 65.87 and 338.73 ± 87.72, respectively indicating no significant difference in the affinity of ParA1 to these dsDNA substrates (Fig. 3B). Nearly similar binding affinity of ParA1 to different types of dsDNA substrates might suggest that ParA1 does not have sequence preference and binds to dsDNA substrates non-specifically. The nonspecific interaction of ParA with dsDNA has been reported in other cases also and shows that this interaction is a must for genome segregation (Pratto *et al*., 2008).

In order to check the effect of ParB1 on ParA1 characteristics, the recombinant ParB1 was evaluated for its characteristic interaction with *segS3* (Charaka and Misra, 2012). As expected, ParB1 showed binding with *segS3* element (Fig. 4A). When the specificity of ParB1 interaction with *segS3* was ascertained through competition with both specific as well as non-specific dsDNAs, we observed that ParB1 binding to *segS3* was not affected in the presence of up to 80 fold excess molar concentration of nonspecific competitor DNA while it got completely abolished in the presence of unlabeled *segS3* (Figs. 4B and 4C). This confirmed that both



(a)



(b)

(c)

Fig. 1: Homology search and functional domain analysis in ParAs of *Deinococcus radiodurans*. Amino acid sequence of ParA in chromosome I (ParA1), chromosome II (ParA2), megaplasmid (ParA3 and ParA4) of *D. radiodurans* (D.rad) and ParA homologue in *Thermus thermophilus* (Thermophilus Soj) were aligned using on line bioinformatic tools. Alignments showing different functional motifs such as Walker A, Walker A', Walker B and DNA binding motifs in all the proteins are depicted (A). Different ParAs or their homologues showing similarities at amino acid levels were used for constructing phylogenetic tree (B). The 3-D model of ParA1 was deduced based on the X-ray crystal structure attributes of the closest chromosomal ParA homologue (Soj) of *Thermus thermophilus* (C)

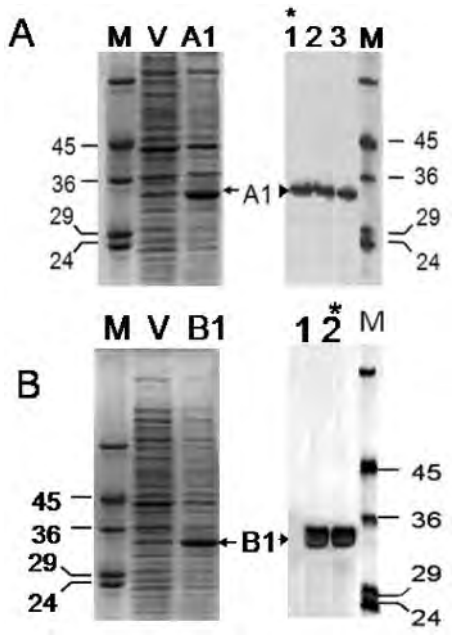


Fig. 2: Expression and purification of recombinant ParA1 and ParB1 of *D. radiodurans*. Transgenic *E. coli* harboring pET28a+ (V) and pET0013 (A1) and pET0012 (B1) were induced with IPTG and expression of ParA1 (A) and ParB1 (B) was observed in cells harboring recombinant plasmids only. Cell free extract from respective cells were used and both the proteins were purified in different fraction (1, 2, 3). Correct sizes of these proteins were judged by comparing with molecular weight markers (M). Fractions marked with asterisk (*) were used in further studies

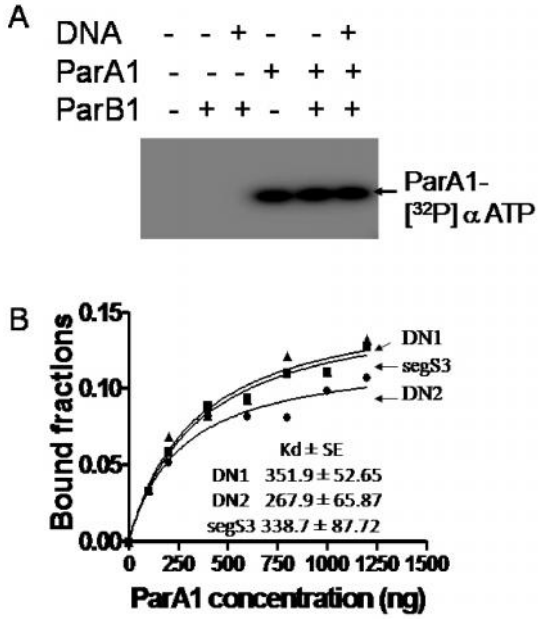


Fig. 3: ATP and DNA binding activity of ParA1. Purified recombinant ParA1 was incubated with [^{32}P] α ATP in the presence and absence of DNA and ParB1 as a negative control. ATP interaction with proteins was trapped by UV cross-linking and detected by autoradiography (A). For DNA binding activity, the different concentration of ParA1 was incubated with Dig labeled *segS*3 (centromere in chromosome I) and 2 non-specific DNA substrates (DN1 and DN2) for 10 min at 37ºC as detailed in materials and methods. Products were analysed on native PAGE and signals were detected by immunoblotting with monoclonal antibodies against Digoxigenin. Signal intensity was quantified densitometrically. Bound fractions were calculated by dividing the nucleoprotein band intensity with the intensity of total substrate in respective sample and plotted as a function of ParA1 concentration (B)

ParA1 and ParB1 are active *in vitro*. Although, both have DNA binding activity, ParB1 is different from ParA1 in terms of its sequence specificity to dsDNA substrates.

ParA1 Dynamics Depend Upon its ATPase Activity and Cognate Elements

ParA/ParA like proteins in other bacteria are shown to form polymers in the presence of ATP and through DNA support. The mechanisms underlying two types of ParAs reported from bacteria are not fully understood. Although, both types of ParAs require ATP for polymerization, their ATPase activity is differentially regulated when encounter to cognate ParB-centromere complexes (Gerdes *et al.*, 2010). Type I oscillates from the poles and polymerizes through DNA, and its depolymerization starts after encountering with ParB bound to centromere. However, in the Type II system, it is believed that ParA polymerization starts from ParB nucleated sites on the genome. Since, the interaction of ParA with ParB-centromere complex in Type I ParAs stimulates its ATPase activity and produces ParA-ADP complexes, ATP hydrolysis manifesting depolymerization is therefore, believed. In this study, since ParA1 is closer to Type I ParA ATPase, the effect of ATP hydrolysis, ratio of ParA1 to ParB1 and ATPase activity stimulation on polymerization/ depolymerization dynamics of ParA1 was examined. ParA1 was incubated with *segS3* in the presence of ADP and both hydrolysable and non-hydrolysable ATP substrates and polymerization if any, was

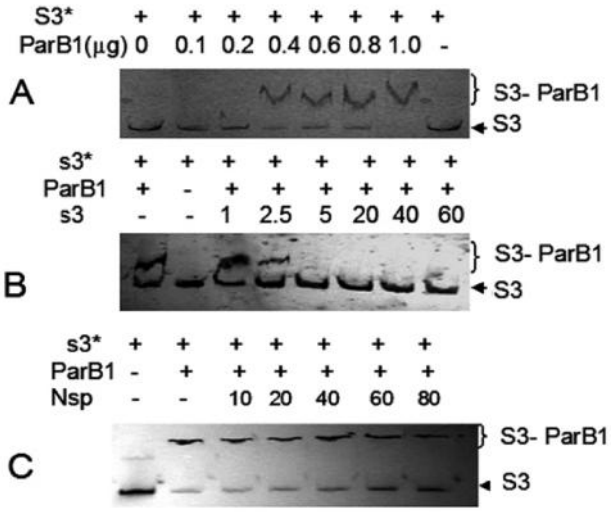


Fig. 4: DNA substrate specificity of purified recombinant ParB1. The dig-labeled *segS3* (S3*) centromere was incubated with increasing concentration of ParB1 (A). Similarly, 500ng of ParB1 was incubated with Dig labeled probe for 10 min followed by addition of an increasing molar concentrations (1-60 fold) of same cold *segS3* (S3) in (B) and 10-80 fold higher amount of DN1 (Nsp) in (C). Mixtures were separated on native PAGE and DNA signal was detected by immunoblotting using Digoxigenin antibodies

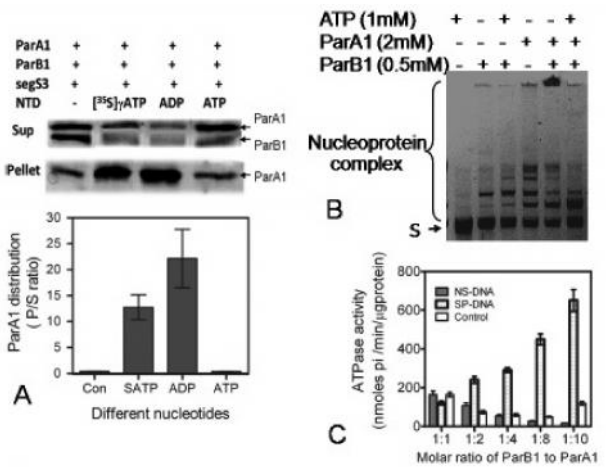


Fig. 5: Functional interaction of ParA1, ParB1 and centromere *in vitro*. Polymerization of ParA1 was determined by sedimentation in the presence and absence of ParB1, *segS3* and nucleotides (NTD). Pellet (P) and supernatant (S) of samples incubated with [^{35}S]ATP (SATP), ATP and ADP were analysed on SDS-PGE and amount of ParA1 in the supernatant and pellet of respective samples was quantified densitometrically and the P/S ratio was represented (A). These proteins were incubated with *segS3* present in 200bp dsDNA substrate and the effect of ATP on formation of nucleoprotein complex was monitored on agarose gel (B). Similarly, 100ng of ParB1 and an increasing concentration of ParA1 were coincubated in the presence and absence of *segS3* (SP-DNA) and DN1 (NS-DNA) and levels of specific ATPase activity of ParA1 was measured (C)

analysed by sedimentation. Results showed a different distribution pattern of this protein in pellet and supernatant (Fig. 5). Here, the amount of ParA1 increased phenomenally in pellet in the presence of non-hydrolysable ATP while reduced to control levels in the presence of hydrolysable ATP (Fig 5A). Quite surprisingly, ParA1 incubated with ADP only also showed very high levels of protein in the pellet for the reasons not clear yet. These results indicated that ParA1 forms polymer in presence of ATP/ADP and the hydrolysis of ATP possibly leads to depolymerization of ParA1 polymers *in vitro*. Therefore, the possibility of ParA1 polymerization on ParB1-centromere complex in the absence of ATP and depolymerization in the presence of ATP was tested. The formation of macromolecular complex of ParA1 with *segS3* was examined by gel retardation assay. We observed that both ParA1 and ParB1 could bind to *segS3* element and showed gel retardation, which increased further ParA1was co-incubated with ParB1-*segS3* complex (Fig. 5B). ATP addition however, resulted into the faster migration of the nucleoprotein complex than migration observed in the absence of ATP. This could be accounted to the conversion of larger complex to smaller size perhaps due to dissociation of ParA1 from this complex and that might indicate the possibility of ATP hydrolysis causing the release of ParA1 from nucleoprotein complex.

Earlier (Charaka and Misra, 2012), we had observed that ParA1 incubated with both ParB1 and *segS3* element showed ATPase activity stimulation. In order to check if stimulation of its ATPase activity also requires dsDNA, we monitored its ATPase activity at the increasing concentration of ParA1 and at a fixed amount of ParB1 in the presence non-specific dsDNA. We noticed that specific activity of ParA1 decreased drastically as we increased its concentration in the absence and presence of dsDNA (Fig. 5C). This observation was against the earlier findings, where levels of ATPase activity was stimulated in presence of both ParB1 and *segS*3

element (Charaka and Misra, 2012). The possibility of dsDNA specificity to ParB1 in stimulation of ParA1 ATPase activity was therefore, hypothesized and ATPase activity of ParA1 was monitored with increasing concentration of ParA1 in presence of *segS3* and ParB1. Interestingly we observed that when the ratio of ParB1 to ParA1 was increased in the presence of *segS3*, the ATPase activity was also stimulated by ~6 times (Fig. 5C). These results indicated that ATPase activity of ParA1 is stimulated only when ParB1 in bound to its cognate centromere like *segS3* and stimulation of ATPase activity is most likely the cause of its depolymerization *in vitro*.

Cellular Dynamics of ParA1 Require its Cognate Elements

In order to understand *in vivo* dynamics of ParA1, GFP-ParA1 chimeric protein was expressed in *E. coli* on pDSWCIO and expression of this chimera was confirmed by immunoblotting (Fig. 6). *E. coli* cells harboring pDAGS3 (*segS3* cloned in mini F instable plasmid pDAG203) were co-expressed GFP-ParA1 and ParB1 on pDSWCIO, and examined under fluorescence microscope at different growth time intervals. These cells produced fluorescent foci of GFP-ParA1 and showed the dynamic movements of such green fluorescent spots in the cells (Fig. 7A). The control cells expressing either GFP-ParA1 alone or GFP in the presence of pDAGS3 showed diffused green fluorescence throughout the cells. This suggested that ParA1 polymerization/ depolymerization dynamics *in vivo* also requires ParB1 and its cognate centromere.

Both wild type and *parB1* mutant of *D. radiodurans* were transformed with pGFPA1 and these cells were induced with IPTG, and the expression of GFP-ParA1 was confirmed by immunoblotting using anti-GFP serum (data not shown). These cells were imaged under fluorescence microscope at different time interval during growth on microscopic slides. We observed that the green fluorescent spot in wild type shows some movement from periphery as the cells progressed towards division and stabilized in the middle of the cell. When these cells have divided, both the daughter cells have acquired green fluorescent spots (Fig. 7B). Such kind

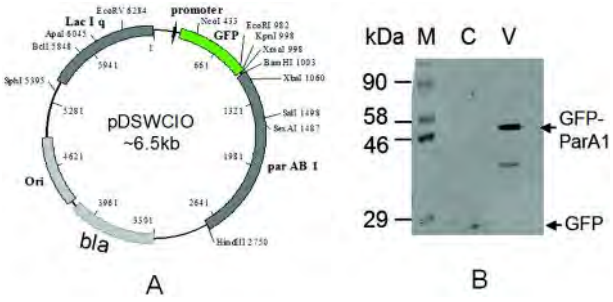


Fig. 6: Construction and expression of GFP translation fusion with ParA1. The *parA1B1* operon in chromosome I of *D. radiodurans* was PCR amplified and cloned downstream to GFP in pDSW209 and resultant plasmid pDSWCIO was obtained (A). Total proteins from recombinant cells harboring expression vector pVHS559 (C) and pVGFPA1 (V), and induced with 10mM IPTG were separated on SDS-PAGE and blotted on PVDF membrane. Expression of recombinant GFP-ParA1 on pVGFPA1 plasmid was confirmed by immunoblotting using antibodies against GFP (B)

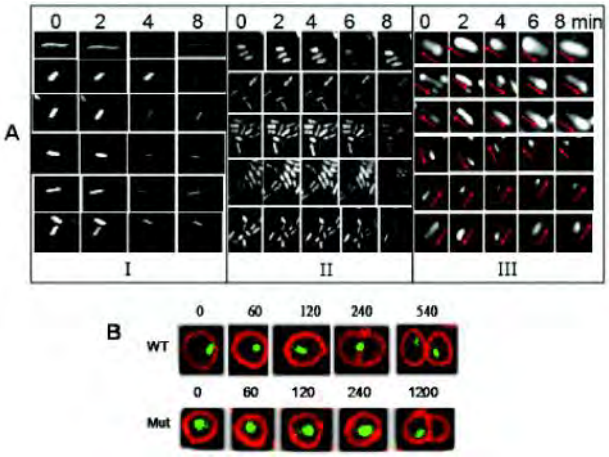


Fig. 7: Fluorescence microscopic studies of bacteria expressing GFP-ParA1. Time lapse fluorescence microscopy was carried out for *E. coli* cells harboring pDAG203 (I) and pDAGS3 (II, III) and expressing both GFP-ParA1 and ParB1 together (I, III) and only GFP-ParA1 (II) under IPTG inducible promoter as described in materials and methods. These cells were grown on microscopic slides and GFP fluorescence was examined under fluorescence microscope for 0-8 min. Arrows indicate the direction of GFP-ParA1 movement during growth of *E. coli* (A). Similarly, *D. radiodurans* wild type (WT) and its *parB1* mutant (Mut) cells expressing both GFP-ParA1 on pVGFPA1 under IPTG inducible promoter were allowed to grow on microscopic slide in the presence of 10mM IPTG and examined periodically under fluorescence microscope for 0- 540 min in wild type and 0-1200 min in case of *parB1* mutant (B). Original picture for both *E. coli* and *D. radiodurans* were recorded at 2µ and 5µ scales respectively, and processed using Adobe Photoshop software

of typical vector movement of green spot was not evident in *parB1* mutant. Furthermore, only one of the two daughter cells is seen carrying green fluorescent spot and other did not show GFP-ParA1 foci. The possibility of cells lacking GFP foci are also nucleated in *parB1* mutant cannot be ruled out (Fig. 7B). Higher frequency of anucleate cells in *parB1* mutant has been reported earlier (Charaka and Misra, 2012). These results might support that *in vivo* dynamics of ParA1 also require its cognate ParB1 and centromere.

The cell division and faithful segregation of genome are tightly linked and regulated processes (Wu and Errington, 2004). In prokaryotes, the mechanisms underlying these processes have been studied mostly in bacteria harboring single circular chromosome and low copy plasmids (Gerdes *et al.*, 2010). Existence of multiple genome system and ploidy has been observed mostly in those bacteria that could tolerate relatively higher levels to biotic and/or abiotic stresses. Molecular basis if any that supports the co-existence of stress tolerance and genome multiplicity in these prokaryotes are not known. However, it is observed that chromosome I in these bacteria encodes functional complements responsible for its normal growth while secondary genome elements contribute largely to stress tolerance and to the mechanisms of cellular responses to stresses. *D. radiodurans* is one amongst the microbes that has multipartite genome and extremely resistant to γ radiation and DNA damage. Therefore, the understanding of molecular mechanisms underlying the multipartite genome maintenance and its correlation with its extreme phenotype has been one of our interests in microbial genome biology. Here we have brought forth some interesting findings to suggest that chromosome I of *D. radiodurans* encodes independent machinery for its partitioning and further demonstrated that ParA1 an essential component of chromosome partitioning system, undergoes polymerization/depolymerization dynamics in the presence ParB1 and its cognate centromere. Further, we observed that the level of ATPase activity seems to be critical for the dynamics of ParA1 *in vitro*. Near absence of GFP-ParA1 dynamics in cells lacking cognate ParB1, in spite of the presence of ParBs encoded on other elements might indicate that ParAs interaction in this bacterium is also restricted to its cognate elements. This conclusion got supported from the results where *E. coli* cells expressing GFP-ParA1 and ParB1 but missing either centromere or ParB1, did not show cellular dynamics of GFP-ParA1. These results suggested that ParA1 of this bacterium require ParB1 and cognate centromere for both *in vitro* and *in vivo* polymerization and depolymerization dynamics, which is required for separation and segregation of duplicated genome, one of the pre-requisite of cell division in bacteria.

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