DNA-Binding Properties of YbaB, a Putative Nucleoid-Associated Protein From Caulobacter crescentus

Parul Pal¹, Malvika Modi¹, Shashank Ravichandran², Ragothaman M. Yennamalli²† and Richa Priyadarshini¹**

¹ Department of Life Sciences, School of Natural Sciences, Shiv Nadar University, Gautam Buddha Nagar, India, ² Department of Bioinformatics, School of Chemical and Biotechnology, SASTRA Deemed to be University, Thanjavur, India

DNA-binding proteins present in bacteria play an important role in nucleoid architecture and gene regulation. NAPs affect bacterial nucleoid organization via DNA bending, bridging, or forming aggregates. EbfC is a nucleoid-associated protein identified first in Borrelia burgdorferi, belonging to YbaB/EbfC family of NAPs capable of binding and altering DNA conformation. YbaB, an ortholog of EbfC found in Escherichia coli and Haemophilus influenzae, also acts as a transcriptional regulator. YbaB has a novel tweezer-like structure and binds DNA as homodimers. The homologs of YbaB are found in almost all bacterial species, suggesting a conserved function, yet the physiological role of YbaB protein in many bacteria is not well understood. In this study, we characterized the YbaB/EbfC family DNA-binding protein in Caulobacter crescentus. C. crescentus has one YbaB/EbfC family gene annotated in the genome (YbaB_Cc) and it shares 41% sequence identity with YbaB/EbfC family NAPs. Computational modeling revealed a tweezer-like structure of YbaB_Cc, a characteristic of YbaB/EbfC family of NAPs. N-terminal–CFP tagged YbaB_Cc localized with the nucleoid and is able to compact DNA. Unlike B. burgdorferi EbfC protein, YbaB_Cc protein is a non-specific DNA-binding protein in C. crescentus. Moreover, YbaB_Cc shields DNA against enzymatic degradation. Collectively, our findings reveal that YbaB_Cc is a small histone-like protein and may play a role in bacterial chromosome restructuring and gene regulation in C. crescentus.

Keywords: DNA binding protein, Caulobacter crescentus, nucleoid associated protein, YbaB/EbfC family, gene regulation

INTRODUCTION

Similar to eukaryotic organisms, bacteria also pack their genetic material in a very small space. DNA-binding proteins known as nucleoid-associated proteins (NAPs) play a crucial role in nucleoid structuring and controlling gene expression. Although NAPs are referred to as histone-like proteins (HLPs), they are functionally very distinct from eukaryotic histones. Bacterial cells contain a wide variety of NAPs and their expression in the cell varies with growth phase of the culture. NAPs are small, basic proteins that can have the ability to bind to DNA, either as monomers, dimers, or tetramers or in complex with other DNA modulating proteins (Dorman, 2009;
The DNA binding occurs either through direct physical interaction or indirectly via binding with other accessory proteins (Dorman, 2014).

Major NAPs involved in nucleoid structuring are HU, IHF, H-NS, Lrp, Fis, and Dps. HU is small, basic, non-sequence specific DNA-binding protein, well conserved in eubacteria (Kamashev and Rouviere-Yaniv, 2000; Bahloul et al., 2001). In dimorphic bacterium, Caulobacter crescentus, HU is a 20-kDa heterodimer consisting of HU1 and HU2 subunits (Le et al., 2013; Le and Laub, 2016). HU protein colocalizes with the nucleoid and has uniform distribution among swarmer and stalked cells but is highly clustered in predivisional cells (Lee et al., 2011). Recently, a new NAP, GapR, was discovered in C. crescentus (Ricci et al., 2016; Arias-Cartin et al., 2017; Taylor et al., 2017). GapR binds AT-rich regions of the nucleoid (Ricci et al., 2016) and absence of GapR leads to morphological and cell division defects (Arias-Cartin et al., 2017; Taylor et al., 2017).

A new family of NAPs, EbfC/YbaB, was first discovered in Borrelia (Babb et al., 2006). The structure of EbfC/YbaB homodimer has been described to have a tweezer-like conformation, with tweezer region ascribed to alpha-helical DNA-binding domain and giving spacer region the ability to fit around double-stranded DNA (Lim et al., 2002; Riley et al., 2009). EbfC protein binding facilitates DNA bending (Riley et al., 2009). In B. burgdorferi, EbfC displays sequence specific DNA binding and binds to a palindromic DNA sequence, 5′-GTnAC-3′, where “n” can be any nucleotide (Riley et al., 2009). However, the DNA binding sites EbfC/YbaB orthologs of E. coli and H. influenzae are not known and are believed to be distinct from that of B. burgdorferi EbfC (Cooley et al., 2009). While EbfC/YbaB is almost present in all eubacteria, its physiological role in most bacterial species remains largely unknown. Here, we characterized YbaB<sub>Cc</sub>, the homolog of EbfC/YbaB family DNA-binding protein in C. crescentus. YbaB<sub>Cc</sub> protein shares 41% sequence identity and has distinctive tweezer-like conformation of YbaB/EbfC family proteins. Moreover, YbaB<sub>Cc</sub> colocalizes with nucleoid and is able to compact DNA. YbaB<sub>Cc</sub> is a non-sequence specific DNA-binding protein with nucleoid-associated function in C. crescentus.

### MATERIALS AND METHODS

#### Strains, Media, and Growth Conditions

C. crescentus strains were grown in peptone yeast extract (PYE) medium or M2G minimal medium at 30°C (Stove Poindexter, 1964) and E. coli cells were grown in LB medium at 37°C. Xylose (0.3%), glucose (0.2%), or arabinose (0.2%) was added to the growth media as required. Plasmids and strains used in this study are detailed in Table 1. Details of strain construction are mentioned in Supplementary Text. All media were purchased from Hi-Media Laboratories (Mumbai, India) and antibiotics were obtained from Sigma (United States).

#### Microscopy and Image Analysis

Expression from PxyI promoter was obtained by addition of xylose to the growth media at an optical density OD<sub>600</sub> 0.2. For studying localization of YbaB<sub>Cc</sub>, expression of CFP-fused YbaB<sub>Cc</sub> was obtained from pXCFPN-5 vector in PB3 temperature-sensitive mutant C. crescentus by addition of xylose (0.2%) at an optical density OD<sub>600</sub> 0.1 in PYE broth. Cultures were incubated at 30°C for 1 h and then shifted to 37°C and grown for 4.5 h. After this, cells were harvested and washed with M2 medium and processed for 4′-6′-diamidino-2-phenylindole (DAPI) staining. Cell samples (5 μl) were imaged as described before (Dubey and Priyadarshini, 2018) using Nikon Eclipse Ti microscope (United States) equipped with Nikon DS-U3 camera. Images were processed with Adobe Photoshop CS6. Cell length, nucleoid length, and cell width were analyzed with Fiji (ImageJ) software (Schindelin et al., 2012) and Oufiti software (Paintdakhi et al., 2015).

#### 4′-6′-diamidino-2-phenylindole Staining

For DNA compaction studies, YbaB<sub>Cc</sub> was ectopically expressed in wild-type E. coli MG1655 cells, as described previously with necessary modifications (Ghosh et al., 2013; Oliveira Paiva et al., 2019). C. crescentus YbaB<sub>Cc</sub> was cloned in pBAD18 and transformed into E. coli MG1655. E. coli cells containing YbaB<sub>Cc</sub> and E. coli control strain carrying empty pBAD18 vector were grown overnight at 37°C in LB medium supplemented with 0.2% glucose and kanamycin. Overnight cells were washed twice with LB broth and secondary cultures were induced with 0.2% arabinose or glucose at OD<sub>600</sub> 0.1. The cells were allowed to grow for 6 h and harvested. Pellets were washed with 1X PBS, fixed in 70% ethanol for 2 min (10% ethanol used in case of colocalization studies with CFP) at room temperature and washed again with 1X PBS. DAPI (1 mg/ml) was added to the cells (1:1,000 dilution) and incubated in dark for 15 min at room temperature. After final wash with 1X PBS, cells were observed by microscopy. For DNA compaction studies, images were captured with Nikon Eclipse Ti 2 Confocal Microscope (AR1MP; United States). Images were processed with Adobe Photoshop CS6. Cell length, nucleoid length, and cell width were analyzed with Fiji (ImageJ) software (Schindelin et al., 2012).

#### Protein Purification

The coding regions of YbaB<sub>Cc</sub> and YbaB<sub>Ec</sub> (Tn) were amplified by PCR and inserted into pET28b(+) (Novagen) between SacI and BamHI sites. These recombinant vectors were used to express 6X His-tagged YbaB<sub>Cc</sub> and YbaB<sub>Ec</sub> (Tn) proteins in E. coli BL21 (DE3) pLysS cells. Recombinant protein expression was obtained by adding 10 μM IPTG to 0.4 OD<sub>600</sub> bacterial culture for 5 h at 37°C in LB medium supplemented with kanamycin. Cells were harvested by centrifugation (10,000 rpm, 5 min, 4°C) and processed for protein purification as described previously with minor modifications (Dubey and Priyadarshini, 2018). The washed pellets were resuspended in the lysis buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5% glycerol, 5 mM imidazole, 1 mM PMSF) and incubated with 1 mg/ml lysozyme for 45 min in shaker incubator at 37°C. Cells were lysed by sonication and the lysate was clarified by centrifugation (16,000 rpm, 20 min, 4°C). The supernatant was treated with DNaseI (New England Biolabs, United States).
TABLE 1 | Strains and plasmids.

| C. crescentus | Relevant genotype or description | Sources/References |
|---------------|---------------------------------|-------------------|
| CB15N         | Synchronizable derivative of wild-type strain CB15 (NA 1000) | Evinger and Agabian, 1977 |
| CJW2141       | CB15N cc2570.pTC67 ftsI(Tes)   | Costa et al., 2008 |
| RP42          | CB15N xylX:pXCFPN-5-ybab<sub>CC</sub> | This work |
| RP43          | CB15N:pPAL4                    | This work |
| RP45          | CB15N cc2570.pTC67 ftsI(Tes) xylX:pXCFPN-5-ybab<sub>CC</sub> | This work |
| E. coli       |                                 |                   |
| DH5α          | φ80 ΔlacZ ΔM15Δ(lacZYA-argF)U169 deoR recA1 endA1ΔhisD17 (rk-::mk +) phoA supE44 thi-1 gyrA96 relA1 | Laboratory strain collection |
| S17           | RP4-2, Tc/Mu, Km-Tn7           | Simon et al., 1983 |
| BL21 (DE3) pLysS | F<sup>−</sup> ompT hsdS<sup>B</sup> (F<sup>−</sup>, m<sup>B</sup>) gal dcm (DE3) pLysS(Cam<sup>R</sup>) | Laboratory strain collection |
| MG1655        | K-12 F<sup>−</sup> λ<sup>−</sup> Δgiv<sup>−</sup> riz-50 rph-1 | Laboratory strain collection |
| RP40          | MG1655/pPAL1                   | This work |
| RP41          | BL21 (DE3) pLysS/pPAL3         | This work |
| RP42          | BL21 (DE3) pLysS/pPAL5         | This work |
| RP46          | MG1655/pBAD18                  | This work |
| Plasmids      |                                 |                   |
| pBAD18        | Kan<sup>+</sup>, DH5α containing empty pBAD18 | Guzman et al., 1995 |
| pXCFPN-5      | TetR, vector used for generating N-terminal protein fusions encoded at the xylX locus | Thanhbichler et al., 2007 |
| pET28b        | Protein expression vector, Kan<sup>+</sup> | Laboratory strain collection |
| pJS14         | High copy number vector, pBR1 MCS derivative, Cmr | Laboratory strain collection |
| pPAL1         | pBAD18 carrying ybab<sub>CC</sub>, Kan<sup>+</sup> | This work |
| pPAL2         | pXCFPN-5 carrying ybab<sub>CC</sub>fused to CFP | This work |
| pPAL3         | pET28b carrying ybab<sub>CC</sub>fused to 6X-His at N-terminus | This work |
| pPAL4         | pJS14 carrying ybab<sub>CC</sub> | This work |
| pPAL5         | pET28b carrying truncated ybab<sub>CC</sub>fused to 6X-His at N-terminus | This work |

for 15 min at 4°C. Supernatant was incubated with Ni<sup>2+</sup>–NTA agarose beads (Qiagen, Germany) equilibrated in binding buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5% glycerol and 10 mM imidazole) for 6 h at 4°C. The mixture was passed through a gravity-flow column and washed with wash buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5% glycerol, and 20 mM imidazole). 6X His-tagged YbaB<sub>CC</sub> was eluted with elution buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5% glycerol, and 300 mM imidazole) in multiple fractions. Eluted fractions were visualized on SDS–PAGE by Coomassie staining (Supplementary Figure 3). Protein containing fractions were pooled and given a buffer exchange for Storage buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5% glycerol), using PD-10 Desalting column (Cytiva, United States). The next day, membranes were given four 10-min washes in PBST and incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Invitrogen, United States) for 1–2 h at room temperature. Blots were washed with PBST and developed with the Bio-Rad Clarity Max ECL Western Blotting Substrates according to the manufacturer’s protocols. 6X His-tagged (Supplementary Figures 3B,C, 4) fusion proteins were confirmed by western blotting for stability.

Western Blot and Dot Blot Analysis

Western blots were performed as described (Dubey and Priyadarshini, 2018). Cell lysates were separated on SDS–PAGE gels and transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer apparatus (Bio-Rad, United States). Dot blot was performed as previously described (Bhat and Rao, 2020), with appropriate modifications. Concentrated and diluted purified YbaB<sub>CC</sub> was spotted onto nitrocellulose membrane and samples were allowed to completely air dry. Membranes were incubated with PBST blocking solution containing 5% non-fat milk at room temperature for 3 h. The membranes were then washed and subsequently incubated with 1:5,000 diluted anti-His antibody (Invitrogen, United States) overnight at 4°C. The next day, membranes were given four 10-min washes in PBST and incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Invitrogen, United States) for 1–2 h at room temperature. Blots were washed with PBST and developed with the Bio-Rad Clarity and Clarity Max ECL Western Blotting Substrates according to the manufacturer’s protocols. 6X His-tagged (Supplementary Figures 3B,C, 4) fusion proteins were confirmed by western blotting for stability.

DNA Protection Assay

To investigate DNA binding and protection ability of YbaB<sub>CC</sub> against degradative action of DNases, pBAD18-kan vector was incubated with increasing YbaB<sub>CC</sub> protein concentrations (20 min, 25°C) in Storage Buffer. The experiments were performed as described earlier (Datta et al., 2019). In brief, enzyme treatment was given at 37°C for 1 min using 1 Unit of DNaseI (New England Biolabs, United States). The enzyme was inactivated by incubation at 75°C for 15 min followed by protein denaturation at 95°C for 20 min. Reactions were electrophoresed
in 1% agarose gel containing ethidium bromide stain and DNA was visualized in a UV transilluminator.

Electrophoretic Mobility Shift Assay
EMSA was performed according to LightShift Chemiluminescent EMSA Kit Protocols, (Thermo Scientific, United States). In brief, oligonucleotides b-WT (124 bp) from *Borrelia burgdorferi* strain B31 (Riley et al., 2009) and cc-WT (200 bp of ybabCc gene) from *C. crescentus* (this work) were labeled at 3′ end with Biotin-11-UTP according to the manufacturer’s protocol (Biotin 3′ end DNA Labeling Kit, Thermo Scientific, United States). Labeled target probes were incubated with increasing concentrations of purified YbaBCc in a reaction containing binding buffer (LightShift Chemiluminescent EMSA Kit, Thermo Scientific) and 50% glycerol at 25°C for 30 min. The reactions were electrophoresed in 6% DNA retardation gel (Invitrogen, United States) in 0.5x TBE. Separated DNA products were then electrophorated onto a positively charged Nylon membrane (Thermo Fisher Scientific, United States) using a semi-dry transfer apparatus (Bio-Rad, United States). Cross-linking by UV was used at 120 mJ/cm² for 1 min immediately after electrophoretic blotting. Detection of DNA and DNA-protein complexes was carried using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific, United States).

Building Computational Model of YbaBCc and Its Complex With DNA
The protein sequence of YbaBCc was retrieved from NCBI (accession id: YP_002515644.1) and was given as input to Robetta tool (Kim et al., 2004), where the comparative modeling approach was used to generate 1,000 sample models and obtained the top five models (Kim et al., 2004). The five models were analyzed to select one model using root mean square deviation as a selection measure. Additionally, the model’s confidence score was also taken into consideration for building the protein-DNA complex. The DNA sequence (5′-ATGAAACGCTGAATGTAAACA-3′) was used to construct a double-stranded B-DNA and the 3D coordinates were obtained using the conformational parameters extracted from fiber-diffraction experimental studies. Subsequently, the protein and DNA were given as input to HADDOCK (van Zundert et al., 2016).

Protein-DNA Docking Studies
The parameters of protein-DNA docking were selected in the manner to perform a blind docking approach, where specific interaction site was not specified (Heté Nyi and van der Spoel, 2002; Teotia et al., 2019; Gondil et al., 2020). Additional parameters were set, such as force constant for center of mass contact restraints (1.0), force constant for surface contact restraints (1.0), radius of gyration (17.78), number of structures of rigid body docking (1,000), number of trials of rigid body minimization (5), sample 180 rotated solutions during rigid body EM (Yes), number of structures for semi-flexible refinement (200), sample 180 rotated solutions during semi-flexible SA (No), Perform final refinement (Yes), number of structures for the final refinement (200), number of structures to analyze (200), Fraction of Common Contacts (FCC) method of clustering method, RMSD cutoff for clustering (0.6), minimum cluster size (4), Non-bonded parameters (OPLX), include electrostatic during rigid body docking (Yes), Cutoff distance to define an hydrogen bind (2.5), cutoff distance to define a hydrophobic contact (3.9), Perform cross-docking (Yes), randomize starting orientations (Yes), perform initial rigid body minimization (Yes), allow translation in rigid body transformation (Yes). The top ranked protein-DNA complex from HADDOCK was analyzed using NUCPLOT (Luscombe et al., 1997).

RESULTS

Protein–DNA Complex Indicates Preferential Binding of YbaBCc
EbfC/YbaB homologs are ubiquitous in nearly all eubacterial genomes. *C. crescentus* genome CCNA_00269 is annotated as DNA-binding protein and BLAST analysis revealed 41% sequence identity and 62% similarity with EbfC/YbaB family NAPs. EbfC/YbaB family of proteins act as NAPs and have dimerization domains flanked on either side by DNA-binding domains (Babb et al., 2006; Riley et al., 2009). CCNA_00269 revealed similar domain arrangement (Figure 1A). Based on these results we annotated CCNA_00269 gene as ybabCc and characterized its DNA binding properties in this study. EbfC/YbaB family of NAPs colocalize with the nucleoid and probably play a role in structuring of the bacterial chromosome (Jutras et al., 2012; Wang et al., 2012). To investigate DNA compaction activity of YbaBCc protein, we introduced full-length YbaBCc on pBAD18 plasmid into *E. coli*. Ectopic expression of YbaBCc was induced by addition of arabinose and cells were subsequently stained with DAPI to label the nucleoid (Figure 1B). As evident from Figures 1B–D, only 51.3% of cell length is occupied by the nucleoid in arabinose-induced cells. In comparison, *E. coli* cells grown with glucose showed 68.4% of the cell length being occupied by nucleoid. Nucleoid compaction observed in presence of glucose could be due to leaky expression from high copy number pBAD18 plasmid (Dubey and Priyadarshini, 2018). In comparison, control RP46 cells carrying empty pBAD18 plasmid grown in presence of arabinose displayed almost 99% of the cell length being occupied by nucleoid. Our results suggest that YbaBCc protein is able to compact DNA.

We further searched for YbaB homologs in other alpha-proteobacteria. YbaB homologs were found in most alpha-proteobacteria except, intracellular pathogens belonging to the family Rickettsiaceae (Figure 2E). *Rhodospirillum rubrum* and *Paracoccus denitrificans* genome were also devoid of YbaB homologs (Figure 2E). EbfC/YbaB family proteins function as homodimers having a unique “tweezer”-like structure (Lim et al., 2002; Cooley et al., 2009). We obtained a 3D model of YbaBCc protein using Robetta program (Kim et al., 2004). Robetta samples nearly 1,000 models obtained after superposing the
**FIGURE 1** | YbaB<sub>cc</sub> binds and compacts DNA in *E. coli*. (A) Schematic presentation of YbaB<sub>cc</sub> protein domain organization. YbaB/EbfC family proteins show similarity in domain structures with YbaB<sub>cc</sub>. There are two DNA-binding domains present at either terminus of YbaB/EbfC family proteins, which together form a unique tweezer-like structure in order to bind to the target DNA. (B) YbaB<sub>cc</sub> overexpression in *E. coli* cells. YbaB<sub>cc</sub> was expressed ectopically from pBAD promoter in RP40 cells. In wild-type *E. coli*, nucleoid occupies the entire cytoplasmic space, whereas a compacted nucleoid tends to be away from the cell periphery and more toward the center of the bacterial cell. The scale bar represents 5 µM. (C) Box and whisker plots of mean cell length. Whiskers represent minimum and maximum cell lengths observed in each strain (1 µM = 32 pixels). Cross (x) represents mean values and horizontal line across boxes represent the median values. Analysis was done using Fiji (ImageJ) software (n, number of cells analyzed = 99 per strain, per condition). (D) Box and whisker plots of mean nucleoid length. Whiskers represent minimum and maximum nucleoid lengths observed in each strain (1 µM = 32 pixels). Cross (x) represents mean values and horizontal line across boxes represent the median values. Analysis was done using Fiji (ImageJ) software (n, number of cells analyzed = 99 per strain, per condition). (C,D) Same cells were analyzed for measuring cell lengths and nucleoid lengths. YbaB<sub>cc</sub> protein bound nucleoid reduces in length as compared to the protein-free nucleoid in non-overexpressing conditions. Box and whisker plots (n = 99 per strain) were generated for statistical analysis with the whiskers including all data points within 1.5*IQR*. ns = non-significant. *p < 0.0001 by one-way ANOVA compared to the empty vector pBAD (ara) control.
**FIGURE 2** | YbaB<sub>cc</sub> is conserved across multiple bacterial species. (A) Multiple sequence alignment of YbaB/EbfC proteins from different bacterial species using MultAlin software (red—high consensus residues, blue—low consensus residues, at the given position). (B) Structural superposition of the modeled YbaB<sub>cc</sub> protein with structural homologs. The modeled protein (colored green and shown in cartoon representation) is a homodimer, as seen in the structural homologs from *E. coli* (PDB id: 1PUG) colored light blue. Another structural homolog from *Haemophilus influenzae* (PDB id: 1J8B) colored magenta is in monomeric form. Image made using PyMOL. (C) Protein-DNA docking model of YbaB<sub>cc</sub> (shown in cartoon and surface representation colored in secondary structure) is observed to bind to the DNA motif (5′-ATGTAACAGCTGAATGTAACAA-3′) as obtained from HADDOCK web server. The insets show the orientation of side chains of the interacting residues in stick representation (colored cyan). (D) Schematic representation of the ybaB/ebfC gene locus with adjacent genes in various bacteria. This arrangement of genes appears to be conserved in most bacteria that harbor YbaB/EbfC proteins. (E) YbaB homologs in alpha proteobacteria. Genomes displaying YbaB homologs with more than 40% sequence identity are labeled in red and proteins having identity below 40% are labeled in orange. Bacterial genomes with no YbaB homologs are labeled in yellow.

Partial threads and gives the user five highly ranked models for a given sequence (Kim et al., 2004). For YbaB<sub>cc</sub> protein sequence, we obtained five models that had a confidence score of 0.75. The confidence score is a qualitative indicator for the user to gauge the success of the modeling, where a confidence score of 0.7 and above for a model is considered as high-quality models that are...
YbaB<sub>Cc</sub> Interaction With DNA Is Not Sequence-Dependent

In order to probe the DNA-binding activity of <i>C. crescentus</i> YbaB, we purified YbaB<sub>Cc</sub> protein, tagged with 6X His at the N-terminal. DNA binding of YbaB<sub>Cc</sub> was first tested using biotin labeled DNA probe corresponding to operator sequences of <i>erpAB</i> in <i>B. burgdorferi</i> (b-WT; Riley et al., 2009). This DNA sequence was selected as EbfC protein from <i>B. burgdorferi</i> preferentially binds to sequences within this region. Oligonucleotide b-WT (124 bp) from <i>Borrelia burgdorferi</i> strain B31 (Riley et al., 2009) was incubated with increasing concentrations (0, 4.7, 14, 23, 32, 42, 45, 47, and 94 μM) of YbaB<sub>Cc</sub> protein for electrophoretic mobility shift assay. YbaB<sub>Cc</sub> protein bound to the duplex DNA probes and formed stable protein-DNA complexes (Figure 4B). EbfC preferentially binds to GTnAC sequence found in <i>erpAB</i> operator 2 region (Riley et al., 2009). To investigate the specificity of YbaB<sub>Cc</sub> binding, we performed EMSA with increasing concentrations of poly (dI-dC) probe as a competitor for non-specific DNA-binding activities. As evident from Figure 4B, concentrations above 2.5 μg of poly (dI-dC) probes (~60-fold higher or above) were able to abolish YbaB<sub>Cc</sub>-DNA complexes, indicating the YbaB<sub>Cc</sub> in <i>C. crescentus</i> might be a non-specific DNA-binding protein. To confirm non-specific DNA-binding activity of YbaB<sub>Cc</sub>, a 200-bp DNA sequence (cc-WT) of <i>ybab</i> gene from <i>C. crescentus</i> was amplified and this biotin-labeled DNA duplex was used as probe in EMSA. Even very low concentrations (14 μM) of YbaB<sub>Cc</sub> protein caused super shift of cc-WT sequence probe (Figure 4C). Taken together, our data suggests that YbaB<sub>Cc</sub> interaction with DNA is not sequence dependent.

Localization of YbaB<sub>Cc</sub> Protein

To determine the cellular localization of the YbaB<sub>Cc</sub> in <i>Caulobacter crescentus</i> cells, YbaB<sub>Cc</sub> was fused with CFP at the N-terminal and expressed from <i>Pxy</i> promoter. RP42 cells showed CFP signal throughout the cell (Supplementary Figure 1). In <i>C. crescentus</i> cells the chromosome spreads from pole to pole making it difficult to differentiate between cytoplasmic and NAPs colocalization with DNA (Arias-Cartin et al., 2017). To probe whether YbaB<sub>Cc</sub> protein colocalized with the nucleoid, the CFP-YbaB<sub>Cc</sub> fusion protein was expressed in a temperature sensitive <i>ftsI</i> <i>C. crescentus</i> mutant, which forms filaments at the restrictive temperature. In this mutant, YbaB<sub>Cc</sub> colocalized with the DAPI signal and was absent in few DNA-free regions (Figure 3), indicating that YbaB<sub>Cc</sub> is a nucleoid associated protein.
Pal et al. DNA Binding Properties of YbaB From Caulobacter crescentus

Figure 4 | YbaB<sub>cc</sub> binds to DNA in a sequence-independent manner in vitro. YbaB<sub>cc</sub> was incubated with 8 nM each of biotin-labeled probes. (A) b-WT (124 bp) from Borrelia burgdorferi strain B31 was incubated with increasing concentrations (0, 4.7, 14, 23, 32, 42, 45, 47, and 94 µM) of YbaB<sub>cc</sub> protein. (C) cc-WT (200 bp) Caulobacter probe was incubated with increasing concentrations (0, 14, 23, 32, and 42 µM) of YbaB<sub>cc</sub> protein. Reactions were electrophoresed on 6% DNA retardation gels and visualized by chemiluminescent detection. YbaB<sub>cc</sub> forms DNA–protein complexes with both probes (A–C) causing shifts of protein–DNA complexes. (B) Competitive binding was also studied by adding varied concentrations (0, 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 µg) of Poly (dI.dC) to YbaB<sub>cc</sub> (32 µM). The presence of free DNA with increasing Poly (dI.dC) indicates sequence independent DNA-binding activity of YbaB<sub>cc</sub> by exchange of b-WT (124 bp) from Borrelia burgdorferi strain B31 probe with Poly (dI.dC). (D) YbaB<sub>cc</sub> (Tn) protein (containing only N-terminal DNA-binding domain) was also tested for its ability to bind b-WT Borrelia probe. However, there was no shift observed even at very high protein concentrations rendering this truncated version of YbaB<sub>cc</sub> to be ineffective in DNA-binding suggesting that both DNA-binding domains are essential for YbaB<sub>cc</sub> protein to bind target DNA.

![Figure 4](image-url)

Using b-WT Borrelia oligonucleotide, YbaB<sub>cc</sub> (Tn) protein was unable to form stable protein-DNA complexes and no shift was observed (Figure 4D). These results suggest that C-terminal DNA-binding domain of YbaB<sub>cc</sub> is important for DNA-binding activity.

YbaB<sub>cc</sub> Protects DNA From Degradation

Most NAPs in bacteria are able to protect DNA from degradation. We investigated YbaB<sub>cc</sub> DNA protection activity by performing DNase I enzymatic degradation assay. Supercoiled pBAD18 plasmid was incubated with increasing YbaB<sub>cc</sub> protein concentrations and then treated with DNaseI enzyme (Figure 5). As seen in Figure 5, supercoiled plasmid DNA was protected from enzymatic degradation in presence of YbaB<sub>cc</sub>. In contrast, the control sample incubated with BSA was degraded upon treatment with DNaseI (Figure 5). Our results indicate that YbaB<sub>cc</sub> protein may protect DNA against damage and degradation.

Overexpression of YbaB<sub>cc</sub> in Nutrient Limiting Conditions Leads to Morphological Aberrations

Abundance of NAPs is highly regulated and fluctuations in protein levels cause adverse effects on cells (Ali Azam et al., 1999). Constitutive overexpression of GapR at high levels is lethal for C. crescentus (Ricci et al., 2016). To investigate
is a sequence specific NAP and acts as a global regulator of gene expression. However, YbaB_Cc and YbaB_Hi do not preferentially bind \textit{B. burgdorferi} palindromic DNA sequence (Cooley et al., 2009) EbfC homolog from \textit{D. radiodurans} also acts as a non-specific DNA-binding protein (Wang et al., 2012). Our results indicate that YbaB_Cc might act as a non-specific DNA-binding protein. Differences in DNA binding are attributed to amino-acid differences in the putative DNA-binding domain (Cooley et al., 2009). \textit{C. crescentus} YbaB_Cc exhibits characteristic tweezer-like conformation of EbfC/YbaB family of NAPs and probably functions as a homodimer. Both N and C-terminals of YbaB_Cc protein have DNA-binding domains and deletion of the C-terminus DNA-binding domain is sufficient to abolish DNA-duplex binding activity (Figure 4D).

Similar to \textit{B. burgdorferi} and \textit{D. radiodurans}, YbaB_Cc is associated with the nucleoid in \textit{C. crescentus} (Jutras et al., 2012; Wang et al., 2012; Figure 3). Ectopic expression of YbaB_Cc in \textit{E. coli} condensed the nucleoid suggesting involvement of YbaB_Cc in nucleoid organization and DNA structuring in cells. Collectively our data indicates that YbaB_Cc is a homolog of EbfC/YbaB and has histone-like activity in \textit{C. crescentus}.

NAPs are involved in global gene expression by making alterations in DNA structure or by interactions with transcriptional machinery. HU is one of the most abundant NAP in bacteria involved in nucleoid structuring (Shindo et al., 1992; Ghosh and Grove, 2004; Nguyen et al., 2009; Oberto et al., 2009; Berger et al., 2010). Surprisingly, \textit{C. crescentus} hu deletion mutants deletion mutants display no adverse effect on cell growth, fitness and chromosome architecture (Christen et al., 2011; Lee et al., 2011). Moreover, loss of other NAPs such as IFH and DPS has no fitness cost on \textit{C. crescentus} indicating functional redundancy among NAPs (Christen et al., 2011). Thus, it is not surprising that YbaB_Cc is not essential for growth and viability of \textit{C. crescentus} under standard laboratory conditions (Christen et al., 2011). While ebfC is an essential gene in \textit{B. burgdorferi} (Riley et al., 2009; Jutras et al., 2012), deletion of ebfC homolog from \textit{D. radiodurans} does not affect cell viability (Wang et al., 2012). However, \textit{D. radiodurans dr0199} mutants have increased sensitivity to UV radiation and oxidative stress (Wang et al., 2012).

Role of NAPs in DNA protection is well established. HU from \textit{Thermotoga maritima} and \textit{Helicobacter pylori} shield DNA from endolytic cleavage by DNase I and hydroxyl radical-mediated damage (Mukherjee et al., 2008; Almarza et al., 2015). Lsr2 from \textit{Mycobacterium tuberculosis} acts against reactive oxygen intermediates by directly binding to DNA and shielding the DNA from damage (Colangeli et al., 2009). EbfC homolog from \textit{D. radiodurans} protects double-stranded DNA from digestion and damage from reactive oxygen species (Wang et al., 2012). Similarly, our results reveal that YbaB_Cc protects double-stranded DNA from enzymatic degradation, probably by direct protein-DNA binding (Figure 5). YbaB_Cc may play a crucial role in maintaining genomic integrity by restricting DNA damage in \textit{C. crescentus}.

Most sequenced eubacterial genomes contain adjacent \textit{dnaX} and \textit{ebfC} genes (Flower and Mchenry, 1990; Flower and McHenry, 1991; Chen et al., 1993). In \textit{B. burgdorferi} and \textit{E. coli}, \textit{dnaX} and \textit{ebfC} are arranged in an operon and are co-transcribed.
FIGURE 6 | YbaB<sub>Cc</sub> overexpression in wild-type C. crescentus. (A) Overexpression of YbaB<sub>Cc</sub> leads to morphological defects in C. crescentus. Expression of YbaB<sub>Cc</sub> from plasmid pJS14 was induced by the addition of 0.3% xylose at an OD of 0.2 and cells were visualized at the indicated time points by Phase Contrast Microscopy (left panel). All images were taken at 100X magnification and optical zoom 1.5X (Scale bar: 2 µm). (B) Distribution of cell length in populations of YbaB<sub>Cc</sub>-overexpressing cells. Micrographs of the strains described in (A) were subjected to automated image analysis. Cell lengths were determined by Oufti Software. Box and whisker plots (n = ±210 per time point and strain) were generated for statistical analysis of imaging data with the whiskers including all data points within 1.5*IQR. Cross (x) represents mean values. **p < 0.0001 by one-way ANOVA compared to the glucose-induced control cells.

together (Riley et al., 2009). C. crescentus genome also has dnaX gene adjacent to YbaB<sub>Cc</sub> (Figure 2D). The dnaX gene encodes the tau and gamma subunits of DNA polymerase and YbaB is a NAP with ability to compact and protect DNA (Flower and McHenry, 1991; Chen et al., 1993). Transcriptional linkage of dnaX and ybaB/ebfC may allow bacterial cells to respond to DNA damage in a timely and regulated manner. Our preliminary experiments to detect transcriptional linkage between dnaX and YbaB<sub>Cc</sub> did not yield positive results (data not shown). This could be due to low levels of transcripts, as the expression of these genes is growth phase-dependent, or dnaX and YbaB<sub>Cc</sub> maybe transcribed independently in C. crescentus. EbfC levels are highest in exponential phase and rapidly decline in stationary phase in B. burgdorferi (Jutras et al., 2012). RecR protein plays crucial role in DNA recombination and repair is also located on the same locus (Figure 2D; Mahdi and Lloyd, 1989; Yeung et al., 1990). Similar to YbaB<sub>Cc</sub>, RecR too is expendable for growth under normal laboratory conditions in C. crescentus (Christen et al., 2011). It is interesting to speculate that both YbaB and RecR may play a role in DNA repair and protection during stress conditions. YbaB homologs are present in most alpha proteobacteria, suggesting an important physiological role of this NAP. Further studies are necessary to elucidate the physiological role of YbaB<sub>Cc</sub> in C. crescentus.

AUTHOR CONTRIBUTIONS

RP and PP designed and conceptualized the study. PP and MM performed the experiments. SR and RY performed the bioinformatics analysis. All authors contributed toward the writing of the manuscript.

FUNDING

PP was supported by UGC-NET-JRF and MM was supported by Inspire-Doctoral fellowship. RP lab was supported by CSIR-EMR grant and start-up funds from Shiv Nadar University. Authors also thank SNU for the use of DST-FIST funded Confocal Imaging facility. SR and RY acknowledge SASTRA Deemed to be University for the infrastructural support and research facilities.

ACKNOWLEDGMENTS

We thank Dr. Karthik Krishnan and the members of the RP lab for their helpful comments and suggestions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.733344/full#supplementary-material
REFERENCES

Ali Azam, T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. (1999). Growth phase-dependent variation in protein composition of the Escherichia coli nucleoid. J. Bacteriol. 181, 6361–6370.

Almarza, O., Nunez, D., and Toledo, H. (2015). The DNA-binding protein Hu has a regulatory role in the acid stress response mechanism in Helicobacter pylori. Helicobacter 20, 29–40. doi: 10.1111/hel.12171

Arias-Cartín, R., Dobhal, G. S., Campos, M., Surovtsev, I. V., Parry, B., and Jacobs-Wagner, C. (2017). Replication fork passage drives asymmetric dynamics of a critical nucleoid-associated protein in Caulobacter. EMBO J. 36, 301–318. doi: 10.15252/embj.201695513

Babb, K., Bykowski, T., Riley, S. P., Miller, M. C., Demoll, E., and Stevenson, B. (2006). Borreli burgdorferi Ebc, a novel, chromosomally encoded protein, binds specific DNA sequences adjacent to erp loci on the spirochete's resident cp32 prophages. J. Bacteriol. 188, 4331–4339. doi: 10.1128/JB.00005-06

Bahloul, A., Boubrik, F., and Rouviere-Yaniv, J. (2001). Roles of Escherichia coli histone-like protein Hu in DNA replication: Hu-beta suppresses the thermostensitivity of dnaA46ts. Biochimie 83, 219–229. doi: 10.1016/S0006-3050(00)00148-2

Berger, M., Farcas, A., Geertz, M., Zhelyazkova, P., Brix, K., Travers, A., et al. (2010). Coordination of genomic structure and transcription by the main bacterial nucleoid-associated protein Hu. EMBO Rep. 11, 59–64. doi: 10.1038/embor.2009.232

Bhat, A. I., and Rao, G. P. (2020). “Dot-blot hybridization technique,” in Characterization of Plant Viruses: Methods and Protocols, eds A. I. Bhat, and G. P. Rao (New York, NY: Springer US), 303–321. doi: 10.1007/978-1-0716-02032-0

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Chen, K.-S., Saxena, P., and Walker, J. R. (1993). Expression of the Escherichia coli dnaX Gene. J. Bacteriol. 175, 6663–6670. doi: 10.1128/JB.175.20.6663-6670.1993

Christen, B., Abelius, E., Collier, J. M., Kalogeraki, V. S., Passarelli, B., Coller, J. A., et al. (2011). The essential genome of a bacterium. Mol. Syst. Biol. 7:528. doi: 10.1038/msb.2011.58

Colangeli, R., Haq, A., Arcus, V. L., Summers, E., Magliozzo, R. S., McBride, A., et al. (2009). The multifunctional histone-like protein La2 protects mycobacteria against reactive oxygen intermediates. Proc. Natl. Acad. Sci. U.S.A. 106, 4414–4418. doi: 10.1073/pnas.0810216106

Cooley, A. E., Riley, S. P., Krai, K., Miller, M. C., DeMoll, E., Fried, M. G., et al. (2009). DNA-binding by Haemophilus influenzae and Escherichia coli YbaB, members of a widely-distributed bacterial protein family. BMC Microbiol. 9:137. doi: 10.1186/1471-2180-9-137

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 16, 10881–10890. doi: 10.1093/nar/16.22.10881

Costa, T., Priyadarshini, R., and Jacobs-Wagner, C. (2008). Localization of PBP3 in Caulobacter crescentus is highly dynamic and largely relies on its functional transpeptidase domain. Mol. Microbiol. 70, 634–651. doi: 10.1111/j.1365-2958.2008.06432.x

Datta, C., Jha, R. K., Kanguly, S., and Nagaraja, V. (2013). Regulation of lipid biosynthesis, HupA (Rv0430), a novel nucleoid-associated protein that regulates a virulence operon in Mycobacterium tuberculosis. J. Bacteriol. 195, 1769–1778. doi: 10.1128/JB.00005-12

Jutras, B. L., Bowman, A., Brissette, C. A., Adams, C. A., Verma, A., Chenail, V. S., Dube, T., Panda, J. J., Yennamalli, R. M., Harjai, K., and Chhibber, S. (2010). Comprehensive evaluation of chitosan nanoparticle based phage lysin delivery system; a novel approach to counter S. pneumoniae infections. Int. J. Pharm. 373, 118850. doi: 10.1016/j.ijpharm.2009.118850

Kamashev, D. M., and Rouviere-Yaniv, J. (1994). The histone-like protein Hu binds specifically to DNA recombinase and repair intermediates. EMBO J. 19, 6527–6535. doi: 10.1093/embob/19.23.6527

Kim, D. E., Chivian, D., and Baker, D. (2004). Protein structure prediction and analysis using the Robetta server. Nucleic Acids Res. 32, W526–W531. doi: 10.1093/nar/gkh468

Le, T. B., and Laub, M. T. (2016). Transcription rate and transcript length drive formation of chromosomal interaction domain boundaries. EMBO J. 35, 1582–1595. doi: 10.15252/embj.201593561

Lee, S. F., Thompson, M. A., Schwartz, M. A., Shapiro, L., and Moerner, W. E. (2011). Super-resolution imaging of the nucleoid-associated protein Hu in Caulobacter crescentus. Biophys. J. 100, L31–L33. doi: 10.1016/j.bpj.2011.02.022

Liu, K., Tempczyk, A., Parsons, J. F., Bonander, N., Toedt, J., Kelman, Z., et al. (2002). Structure note crystal structure of YbaB from Haemophilus influenzae (HI1042), a protein of unknown function coexpressed with the recombinational DNA repair protein RecR. Proteins 50, 375–379.

Luscombe, N. M., Laskowski, R. A., and Thornton, J. M. (1997). NUCPLOT: a program to generate schematic diagrams of protein-nucleic acid interactions. Nucleic Acids Res. 25, 4940–4945. doi: 10.1093/nar/25.24.4940

Mahdi, A. H., and Lloyd, G. R. (1989). The recR locus of Escherichia coli K-12: molecular cloning. DNA sequencing and identification of the gene product: thi eRcso shri oh1K1mlc11nmsm DAsq cmn n dniiano h. Nucleic Acids Res. 17, 6781–7974.

Mukherjee, A., Sokumbi, A. O., and Grove, A. (2008). DNA protection by histone-like protein Hu from the hyperthermophilic eubacterium Thermotoga maritima. Nucleic Acids Res. 36, 3956–3968. doi: 10.1093/nar/gkn348

Nguyen, H. H., de la Tour, C. B., Toueille, M., Sommer, S., and Servant, P. (2009). The essential histone-like protein Hu plays a major role in Deinococcus radiodurans nucleoid compaction. Mol. Microbiol. 73, 240–252. doi: 10.1111/j.1365-2958.2009.06766.x

Oberto, J., Nabati, S., Jooste, V., Mignot, H., and Rouviere-Yaniv, J. (2009). The Hu regulon is composed of genes responding to anaerobiosis, acid stress, high osmolality and SOS induction. PLoS One 4:e4367. doi: 10.1371/journal.pone.0004367

Oliveira Paiva, A. M., Friggen, A. H., Qin, L., Douwes, R., Dame, R. T., and Smits, W. K. (2019). The bacterial chromatin protein HupA can remodel DNA and
associates with the nucleoid in *Clostridium difficile*. *J. Mol. Biol.* 431, 653–672. doi: 10.1016/j.jmb.2019.01.001

Paintdakhi, A., Parry, B., Campos, M., Irnov, I., Elf, J., Surovtsev, I., et al. (2015). Oufit: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. *Mol. Microbiol.* 99, 767–777. doi: 10.1111/mmi.13264

Ricci, D. P., Melfi, D. M., Lasker, K., Dill, D. L., McAdams, H. H., and Shapiro, L. (2016). Cell cycle progression in *Caulobacter* requires a nucleoid-associated protein with high AT sequence recognition. *Proc. Natl. Acad. Sci. U.S.A.* 113, E5952–E5961. doi: 10.1073/pnas.1612579113

Riley, S. P., Bykowski, T., Cooley, A. E., Burns, L. H., Babb, K., Brissette, C. A., et al. (2009). *Borrelia burgdorferi* EbfC defines a newly-identified, widespread family of bacterial DNA-binding proteins. *Nucleic Acids Res.* 37, 1973–1983. doi: 10.1093/nar/gkp027

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Shindo, H., Furubayashi, A., Shimizu, M., Miyake, M., and Imamoto, F. (1992). Preferential binding of *E.coli* histone-like protein HU alpha to negatively supercoiled DNA. *Nucleic Acids Res.* 20, 1553–1558. doi: 10.1093/nar/20.7.1553

Simon, R., Priefer, U., and Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Nat. Biotechnol.* 1, 784–791. doi: 10.1038/nbt1183-784

Song, Y., DiMaio, F., Wang, R. Y.-R., Miles, C., Brunette, T., et al. (2013). High-resolution comparative modeling with RosettaCM. *Structure* 21, 1735–1742. doi: 10.1016/j.str.2013.08.005

Stove Poindexter’, J. (1964). Biological properties and classification of the *Caulobacter* group. *Bacteriol. Rev.* 28, 231–295.

Taylor, J. A., Panis, G., Viollier, P. H., and Marczyński, G. T. (2017). A novel nucleoid-associated protein coordinates chromosome replication and chromosome partition. *Nucleic Acids Res.* 45, 8916–8929. doi: 10.1093/nar/gks596

Teotia, D., Gaid, M., Saini, S. S., Verma, A., Yennamalli, R. M., Khare, S. P., et al. (2019). Cinnamate-CoA ligase is involved in biosynthesis of benzoate-derived biphenyl phytoalexin in *Malus 3 domestica* “Golden Delicious” cell cultures. *Plant J.* 100, 1176–1192. doi: 10.1111/tpj.14506

Thanbichler, M., Iniesta, A. A., and Shapiro, L. (2007). A comprehensive set of plasmids for vanillate-and xylose-inducible gene expression in *Caulobacter crescentus*. *Nucleic Acids Res.* 35:e137. doi: 10.1093/nar/gkm818

van Zundert, G. C. P., Rodrigues, J. P. G. L. M., Trellet, M., Schmitz, C., Kastritis, P. L., Karaça, E., et al. (2016). The HADDOCK2.2 web server: user-friendly integrative modeling of biomolecular complexes. *J. Mol. Biol.* 428, 720–725. doi: 10.1016/j.jmb.2015.09.014

Wang, H., Wang, F., Hua, X., Ma, T., Chen, J., Xu, X., et al. (2012). Genetic and biochemical characteristics of the histone-like protein DR0199 in *Deinococcus radiodurans*. *Microbiology* 158(Pt 4), 936–943. doi: 10.1099/mic.0.053702-0

Yeung’ T., Mullin, D. A., Chen, K.-S., Craig, E. A., Bardwell, J. C. A., and Walker’, J. R. (1990). Sequence and expression of the *Escherichia coli* recR locus. *J. Bacteriol.* 172, 6042–6047.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Pal, Modi, Ravichandran, Yennamalli and Priyadarshini. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.