Initiation of Chromosomal Replication in Predatory Bacterium
Bdellovibrio bacteriovorus

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Bdellovibrio bacteriovorus is a small Gram-negative predatory bacterium that attacks other Gram-negative bacteria, including many animal, human, and plant pathogens. This bacterium exhibits a peculiar biphasic life cycle during which two different types of cells are produced: non-replicating highly motile cells (the free-living phase) and replicating cells (the intracellular-growth phase). The process of chromosomal replication in B. bacteriovorus must therefore be temporally and spatially regulated to ensure that it is coordinated with cell differentiation and cell cycle progression. Recently, B. bacteriovorus has received considerable research interest due to its intriguing life cycle and great potential as a prospective antimicrobial agent. Although, we know that chromosomal replication in bacteria is mainly regulated at the initiation step, no data exists about this process in B. bacteriovorus. We report the first characterization of key elements of initiation of chromosomal replication – DnaA protein and oriC region from the predatory bacterium, B. bacteriovorus. In vitro studies using different approaches demonstrate that the B. bacteriovorus oriC (BdoriC) is specifically bound and unwound by the DnaA protein. Sequence comparison of the DnaA-binding sites enabled us to propose a consensus sequence for the B. bacteriovorus DnaA box [5′-NN(A/T)TCCACA-3′]. Surprisingly, in vitro analysis revealed that BdoriC is also bound and unwound by the host DnaA proteins (relatively distantly related from B. bacteriovorus). We compared the architecture of the DnaA–oriC complexes (orisomes) in homologous (oriC and DnaA from B. bacteriovorus) and heterologous (BdoriC and DnaA from prey, Escherichia coli or Pseudomonas aeruginosa) systems. This work provides important new entry points toward improving our understanding of the initiation of chromosomal replication in this predatory bacterium.

Keywords: oriC, DnaA, initiation of chromosome replication, Bdellovibrio bacteriovorus, Escherichia coli, Pseudomonas putida

INTRODUCTION

Bdellovibrio are small intriguing Gram-negative predatory bacteria that enter and kill other Gram-negative bacteria, including many pathogens, such as Campylobacter, Helicobacter (Markelova, 2010), Escherichia (Varon and Shilo, 1968), Pseudomonas, Salmonella (Iebba et al., 2014), Pusobacterium nucleatum, and Aggregatibacter actinomycetemcomitans (a member of oral microbial communities) (Loozen et al., 2015). The widespread species of this genus is Bdellovibrio bacteriovorus, which inhabits a wide range of environments, including fresh water, sewage, soil, and even mammalian intestines (Rendulic et al., 2004). B. bacteriovorus is a small bacterium...
(0.2–0.5 µm wide and 0.5–2.5 µm long) that possesses a relatively large 3.85-Mb genome that encodes many predation-associated proteins, such as proteases, peptidases, and other hydrolytic enzymes.

*Bdellovibrio bacteriovorus* exhibits a biphasic lifecycle consisting of a free-living non-replicative attack phase and an intracellular growth phase (Sockett, 2009). In the free-living phase, this highly motile bacterium searches for its prey; after attaching to the prey's outer membrane, it passes through the peptidoglycan layer into the periplasm and begins its intracellular growth phase (Lambert et al., 2008). Inside the periplasm, *B. bacteriovorus* degrades the host's macromolecules using different types of hydrolytic enzymes, allowing it to grow and replicate its chromosome (Rendulic et al., 2004). This chromosomal replication is not followed by cell division, but instead leads to the formation of a multinucleoid elongated filamentous. When the resources of the host cell are exhausted, the elongated filament synchronously septates to form usually three to six *B. bacteriovorus* progeny cells (Fenton et al., 2010). These progeny cells become motile, and then are released into the environment through lysis of the host cell. Interestingly, *B. bacteriovorus* can also enter (albeit rarely and only in the presence of abundant amino acids and cofactors) into a replicative host-independent phase (Seidler and Starr, 1969). *B. bacteriovorus* has received considerable recent research interest, owing to its intriguing life cycle and its great potential to be applied as an antimicrobial agent in industry, agriculture, and/or medicine. To fully utilize *B. bacteriovorus* in any of these roles, however, we must better understand the cell biology of this pathogen at the molecular level.

Chromosomal replication, which is a key event in the bacterial life cycle, is mainly controlled at the initiation step (Zakrzewska-Czerwińska et al., 2007). In *B. bacteriovorus*, as in other bacteria, the initiation of chromosomal replication is strictly regulated and adjusted with respect to its cell cycle. Replication must be initiated after *B. bacteriovorus* enters the prey, and it must cease before bdelloplast septation to ensure that each cell receives a single copy of the chromosome. However, even the key elements of replication initiation have not yet been identified for *B. bacteriovorus*.

In bacteria, replication begins at a single chromosome site called the origin of replication (oriC). The process is initiated through the cooperative binding of the initiator protein, DnaA, to specific 9-mer sequences (called DnaA boxes) within the oriC region. This causes the DNA strands to separate at the AT-rich DNA unwinding element (DUE), allowing the entry of helicase and, later, other enzymes required for DNA synthesis (e.g., primase and DNA Pol III). Bacterial origins, which may be a continuous unit or divided in two parts (bipartite oriC), range in length from ~200 to 1000 bp or longer (when they are split). They can differ in various characteristics, including the numbers, orientations, and sequences of their DnaA boxes, and the localizations and sequences of the AT-rich regions and other motifs, including those recognized by regulatory proteins. The various modules (e.g., DnaA boxes, the DUE, etc.) constitute the central management system responsible for forming the functional initiation complex (osomes) and/or regulating the assembly of this complex (Leonard and Grimwade, 2015; Wolański et al., 2015).

Here, we report the first characterization of DnaA and oriC from the predatory bacterium, *B. bacteriovorus*. We demonstrate that the *B. bacteriovorus* oriC (BdoriC) is specifically bound and unwound not only by its own DnaA, but surprisingly also by the host's DnaA proteins.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

The wild-type *B. bacteriovorus* strain HD100 (Rendulic et al., 2004) and the axenic *B. bacteriovorus* strain HI (Roschanski et al., 2011) were used in this study. *B. bacteriovorus* HD100 was grown at 30°C by predation on *Escherichia coli* S-17 in HEPES buffer (25 mM HEPES, 2 mM CaCl$_2$, 3 mM MgCl$_2$, pH 7.8) and 200 rpm, or on double-layer plates [bottom layer – YPSC medium (0.1% Yeast Extract, 0.1% Pepton, 0.05% Sodium Acetate, 0.025% Magnesium Sulfate; pH 7.6) with 1% agar, top layer – YPSC with 0.6% agar and supplemented with *E. coli* S-17 liquid culture, both layers were supplemented with 0.025% CaCl$_2$ after autoclaving]. *E. coli* was grown in LB medium (liquid or agar) at 37°C. *B. bacteriovorus* HI was grown in PYE medium (1% Bacto Peptone, 0.3% yeast extract, 2 mM CaCl$_2$, 3 mM MgCl$_2$, pH 7.6) at 30°C and 200 rpm.

**In silico Origin Prediction**

The oriC-type replication origins in the genomes of *B. bacteriovorus* HD100 [GenBank entry BX842601.2], *B. bacteriovorus* str. Tiberius [GenBank entry CP002930.1], *Bdellovibrio exovorus* JSS [GenBank entry CP003537.1], and *Halobacteriovorax marinus* SJ [GenBank entry FQ312005.1] were predicted using the following stepwise procedure: (1) The annotation of the dnaA gene in the genome was validated by TBLASTN (version 2.2.30) (Shiryev et al., 2007) using the DnaA sequence of *E. coli* K-12 MG1655 [GenBank entry AAC76725.1] as a query. (2) The approximate genomic location of oriC was roughly determined based on the inflection point (minimum) of the genome's cumulative GC-skew, which was obtained from the Comparative Genometrics website (Roten et al., 2002) or the GenSkew webservicer with the following parameter settings: Nucleotide1: G, Nucleotide2: C, Windowsize: 500, Stepsize: 100. (3) WebSIDD (Bi and Benham, 2004)$^1$ was used under default settings (37°C, 0.1 M salt, circular DNA, copolymeric) and negative superhelicity values in the range of $\sigma = -0.04$ (low) to $\sigma = -0.06$ (high) in increments of 0.005, in order to identify putative DUE(s) (Kowalski and Eddy, 1989) in intergenic regions near (+10 kb) of the GC-skew inflection point (minimum). (4) DnaA boxes were assigned manually using the *E. coli* consensus, 5′-TTTGCACA (Schaper and Messer, 1995), and allowing for three mismatches. (5) A prediction was considered significant if a DnaA box could be assigned to a position of approximately two helical turns distant from the border of a strong DUE.

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1. http://genskew.csb.univie.ac.at/
2. http://benham.genomecenter.ucdavis.edu/sibz/
output data were obtained as raw text files and further processed with Microsoft Excel v97SR-1 and Corel Draw v.11.

**DnaA Purification**
The *B. bacteriovorus dnaA* gene was PCR amplified from chromosomal DNA with primers P-1 and P-2 (Supplementary Table S1), cut with BamHI and XhoI and then cloned into the pET28a(+) expression vector linearized with the same restriction enzymes. The 6HisBdDnaA protein was produced in *E. coli* BL21 containing pET28a(+)BddnaA. When the culture reached an OD_{600} = 1.9, fusion protein synthesis was induced by addition of 1 mM IPTG, after which cells were incubated for 3 h at 37°C. The bacteria were harvested by centrifugation (10 min, 5000 g, 4°C) and the bacterial pellets were stored at −20°C. The purification of 6HisBdDnaA was performed as described previously (Zawilak-Pawlik et al., 2006), except that LG$_{100}$ buffer (45 mM HEPES/KOH, pH 7.6, 100 mM potassium glutamate, 10 mM magnesium acetate, 1 mM DTT and 20% sucrose) was used in place of the LG$_{200}$ buffer.

**Electrophoretic Mobility Shift Assay (EMSA)**
The interactions of the DnaA protein with DNA were analyzed as previously described (Zawilak et al., 2001; Donczew et al., 2015) with minor modifications. The IRD-700-labeled BdoriC fragment (12 fmol, 623 bp, PCR amplified using primers P-5/P-4) was used in place of the LG$_{200}$ buffer.

**P1 Nuclease Assay**
The P1 nuclease assay was performed as previously described (Donczew et al., 2012). The pOCBdoriC plasmid (112 pmol) was incubated with DnaA proteins (0, 17.5, 35, 70, and 140 pmol from *B. bacteriovorus*, *E. coli*, or *P. putida*), and the presence of unwound DNA was examined by P1 treatment followed by digestion with SspI. The digestion products were visualized on 1% agarose gel using a Molecular Imager® Gel DocTM XR+ System and the Image Lab Software (Bio-Rad).

**oriC Activity**
The *E. coli* strains, WM1785 and its polA derivative, WM1838 (polA−, fadA::Tnl0), were used as host strains in the ori assay (Woelker and Messer, 1993). Chemically competent WM1785 and WM1838 cells were heat-shock-transformed using 50 ng of the appropriate plasmid (pBR322, pOCl70, pBR322BdoriC, or pOCBdoriCΔori). The transformed cells were then cultivated on agar plates with tetracycline (12.5 μg/ml; for pBR322 and pBR322BdoriC) or ampicillin (100 μg/ml; for pOCl70 and pOCBdoriCΔori) overnight at 30°C (for WM1838) or 37°C (for WM1785).

**DMS Footprinting and PE Analysis**
DNA modification with dimethyl sulfate (DMS) was performed as previously described (Sasse-Dwight and Gralla, 1991; Donczew et al., 2015). The reaction mixtures (50 μl) contained 25 mM HEPES/KOH, pH 7.6, 12% (v/v) glycerol, 1 mM CaCl$_2$, 0.2 mM EDTA, 5 mM ATP, 0.1 mg/ml BSA, 15 mM pOCBdoriC, and 6HisBdDnaA protein (0, 200, 400, 800, or 1600 nM). After the mixtures were incubated at 30°C for 10 min, 3.6 μl of 150 mM DMS (Sigma) was added to a final concentration of 10 mM, and the incubation was continued for 5 min. The reaction was quenched by the addition of 100 μl of cold Stop Buffer (3 M ammonium acetate, 1 M 2-mercaptoethanol, 20 mM EDTA). The samples were precipitated with cold ethanol, dried, dissolved in 100 μl of 1 M piperidine, and incubated at 90°C for 30 min.

**RIP Mapping**
Replication initiation point (RIP) mapping was performed essentially as previously described (Gerbi and Bielinsky, 1997;
Bielinsky and Gerbi, 1999; Matsunaga et al., 2003; Donczew et al., 2012. B. bacteriovorus cells were grown in 1000 ml HEPES buffer supplemented with P. putida cells (OD<sub>600</sub> = 1.0), and P. putida cells were grown in LB medium (OD<sub>600</sub> = 1.0). B. bacteriovorus were grown until the solution became viscous and slightly clear, whereupon the medium was passed through a 0.45-µm filter and then pelleted. The bacterial pellets were resuspended in 30 ml of TEN buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl) and disrupted by the addition of sodium dodecyl sulfate (SDS) and sodium sarcosyl (final concentration, 1% each). The mixture was subjected to three-step extraction with phenol:chlorormisoamyl alcohol (25:24:1, v/v) and after that 1.1 g/ml CsCl and 6 µl Midori Green Advanced DNA Stain (Nippon) were added to the aqueous phases. The genomic DNA was purified by CsCl gradient ultracentrifugation. To enrich the replication intermediates, the total isolated DNAs (75 µg for B. bacteriovorus and 426 µg for P. putida) were passed through BND-cellulose columns (Sigma-Aldrich) pre-equilibrated with NET buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 M NaCl). The columns were washed with five volumes of NET buffer, and DNA was eluted at 50°C with NET buffer containing 1.8% caffeine. To remove nicked DNA, the recovered DNAs (48 µg for B. bacteriovorus and 58 µg for P. putida) were subjected to phosphorylation by T4 kinase (Thermo Scientific) followed by λ-exonuclease (Thermo Scientific) digestion. The FR reactions contained 1 unit of vent (exo-) DNA polymerase (Thermo Scientific), 0.6 µg of prepared DNA, and 350 fmol of 32P-labeled primer P-19. After 35 cycles of reaction (30 s at 95°C, 30 s at 55°C, and 60 s at 72°C), the amplified products were separated on an 8% polyacrylamide gel under denaturing conditions and analyzed with a Typhoon FLA 9500 Biomolecular Imager (GE Healthcare).

**Immunoprecipitation Assay**

Immunoprecipitation assays were performed as described elsewhere (Jakimowicz et al., 2002). Briefly, B. bacteriovorus HI cells were grown to OD = 1.0 in 40 ml of PYE medium, and then formaldehyde was added to final concentration 1% (v/v) and the samples were incubated for 30 min. Anti-6HisBdDnaA polyclonal antibodies (ProteoGenix) were used to precipitate BdDnaA-DNA nucleoprotein complexes, and PCR was used to amplify regions of interest (primers P-3/P-4 for oriC and primers P-11/P-12 for non-box DNA). The PCR fragments were resolved on 1.5% agarose gels and analyzed using a Gel Doc<sup>TM</sup> XR+ Imaging System (Bio Rad).

**RESULTS**

B. bacteriovorus oriC is Specifically Bound by BdDnaA In vivo and In vitro

To determine whether the in silico-predicted B. bacteriovorus oriC region is bound in vivo by the initiator BdDnaA protein, we performed immunoprecipitation assays using antibodies against the purified 6HisBdDnaA protein (Supplementary Figure S2). The formaldehyde cross-linked BdDnaA-BdoriC complexes formed in B. bacteriovorus grown under host-independent conditions were enriched by affinity chromatography, and the released DNA fragments were identified by PCR (Figure 1A). We obtained strong PCR signals using primers for the BdoriC fragment but not a non-box DnaA fragment (Figure 1B), indicating that BdDnaA-BdoriC complexes were successfully detected.

To examine whether the BdDnaA protein interacts with BdoriC in vitro, we applied electrophoretic mobility shift assays (EMSAs) and SPR. Our EMSAs demonstrated that the BdoriC region, but not the non-DnaA box fragment, was bound specifically by the BdDnaA protein (Figure 1B). Interestingly, although the BdoriC contains eight putative DnaA boxes, only one nucleoprotein complex was observed. Moreover, increasing the protein concentration did not lead to the formation of SIDD, stress-induced DNA duplex destabilization) for localizing the DNA-unwinding element (DUE) (for details see Materials and Methods). Similar to the previous findings of Gao et al. (2013), we obtained predictions for oriC-type replication origins within the ~250-bp-long dnaA-dnaN intergenic regions of the genomes of B. bacteriovorus (HD100 and str. Tiberius strains) and other species belonging to this genus, including Halobacteriovorax marinus SJ and B. exovorus JSS (B. exovorus JSS is not yet included in the DoriC data set created by Gao et al., 2013). The predicted Bdellobivirio oriC region contains eight putative DnaA boxes (see Supplementary Figure S1; Figure 6).

Interestingly, two of the putative DnaA boxes (boxes 7 and 8; Supplementary Figure S1; Figure 6) are usually situated within the dnaA gene, at its 3'-end. The position of the DUE could be readily derived from the SIDD plots generated for all of the analyzed organisms, with the exception of B. exovorus JSS. As seen for other bacterial origins, the first in the cluster of DnaA boxes could be assigned to a position approximately two helical turns distant from the border of a strong SIDD site (see Supplementary Figure S1; Figure 6). In the case of B. exovorus JSS, we were able to assign a “predicted oriC” based on the high similarity of DnaA box distances and orientations in this organism and the two B. bacteriovorus strains for which we were able to predict oriC. In all four genomes, the predicted oriC is flanked upstream by the dnaA, rpmH, rnpA, and yidC (oxaA) genes, and downstream by the dnaN, recF, gyrB, and gyrA genes. This particular oriC gene context is also found in many genomes from the Actinobacteria and Firmicutes (Ogasawara et al., 1985).

In sum, the in silico-predicted B. bacteriovorus oriC region contains origin-characteristic elements (DnaA boxes and a DUE) and is located between the dnaA and dnaN genes within the gene cluster of rnpA-rpmH-dnaA-dnaN-recF-gyrB-gyrA, which is conserved in some bacterial species.
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FIGURE 1 | BdDnaA interacts specifically with BdoriC in vivo and in vitro. (A) In vivo identification of the BdDnaA-BdoriC complexes. Anti-6HisBdDnaA polyclonal antibodies were used to immunoprecipitate BdDnaA-BdoriC complexes cross-linked with formaldehyde. The oriC region and non-box DnaA fragment (negative control) were amplified by PCR (see Materials and Methods). Lanes: IP, immunoprecipitated DNA; and C, total genomic DNA extracted from B. bacteriovorus cells (PCR control). (B) EMSA analysis of the interaction between 6HisBdDnaA and BdoriC in vitro. IRD-700 labeled DNA fragments, BdoriC (635 bp), and control DNA (part of the bd2045 gene, 620 bp) were incubated with increasing amounts of 6HisBdDnaA proteins, and the formed nucleoprotein complexes were analyzed on a 4% polyacrylamide gel. (C) SPR analysis of the interaction between 6HisBdDnaA and BdoriC in vitro. Biotinylated versions of the BdoriC region (652 bp) and a non-box DNA fragment (649 bp) were immobilized on the chip surface (Sensor Chip SA) in a Biacore T200 apparatus. Sensograms were obtained for different concentrations of 6HisBdDnaA interacting with the DNA fragment containing BdoriC. The BIA evaluation 3.0 software was used for data analysis.

additional higher-molecular-weight complexes. The interaction between BdDnaA and BdoriC was also confirmed by SPR analysis (Figure 1C), which showed that the RU values were proportional to the BdDnaA concentration.

Together, these findings indicate that the in silico-predicted B. bacteriovorus oriC is bound specifically by the initiator protein, BdDnaA, in vitro and in vivo.

The BdDnaA Protein Specifically Binds DNA Sequences Corresponding to the In silico-Assigned DnaA Boxes

To further define the in silico-predicted DnaA boxes and gain additional insight into their abilities to bind the BdDnaA protein, we applied footprinting experiments using DMS. This agent primarily methylates deoxyguanosine residues, making the proximate phosphodiester bond susceptible to piperidine cleavage (Maxam and Gilbert, 1977). The pOCBdoriC plasmid, which contained the entire oriC region, was incubated with increasing concentrations of BdDnaA protein, and then subjected to DMS modification and subsequent PE of piperidine-cleaved DNA. We identified 16 nucleotides that exhibited BdDnaA-dependent protection from DMS modification: eight Gs on the upper strand and eight Gs on the lower strand (Figure 2).

Fourteen of them are located within the seven of eight in silico-predicted DnaA boxes, while the remaining two lie between in silico-predicted DnaA boxes (box; Figure 2 and see also Figure 6). Thus, the DMS footprinting confirmed the results of our in silico analysis and allowed us to identify additional DnaA-binding sites. Moreover, this analysis showed that the two unusually located DnaA boxes, boxes 7 and 8 (Figure 6A), are bound by the BdDnaA protein (Figure 2D).

Based on the assumption that, as in other bacteria, the DnaA box sequence of B. bacteriovorus consists of nine nucleotides, we aligned the DNA sequences in the vicinity of the protected nucleotides and obtained a proposed consensus sequence for the BdDnaA-binding motif, 5′-NN(A/T)TCCACA-3′, which we designated the DnaA box (Figure 6B; Supplementary Table S2).

Collectively, these analyses show that BdDnaA specifically binds to eight sites (Figures 2A–D) within the identified BdoriC region of B. bacteriovorus.

DnaA Proteins from Prey Organisms Specifically Bind BdoriC In vitro

Interestingly, the binding mode of the E. coli DnaA protein to the oriC of E. coli (EcorI) (Weigel et al., 1997) appears to
FIGURE 2 | 6HisBdDnaA recognizes specific DNA sequences within BdoriC. DMS footprinting. pOCBdoriC plasmids were incubated with increasing amounts of 6HisBdDnaA proteins (0, 0.175, 0.35, 0.7, 1.4, and 2.8 µM), treated with DMS, and then used as a substrate for primer extension (PE) analysis. (A–D) DMS footprints were obtained with 32P-labeled primers P-10 (A), P-9 (B), P-18 (C), and P-7 (D). Primers P-7, P-9, and P-10 are complementary to the lower strand, while primer P-18 is complementary to the upper strand. Solid lines and dashed arrows indicate the nucleotides of the lower strand and upper strand, respectively, that become sensitive to DMS upon protein binding.

FIGURE 3 | DnaA proteins from host organisms, Escherichia coli and P. putida, interact specifically with B. bacteriovorus oriC. EMSA – an IRD-700-labeled BdoriC fragment (635 bp) was incubated with increasing amounts of DnaA proteins from E. coli (EcDnaA) or P. putida (PpDnaA), and the nucleoprotein complexes were analyzed on a 4% polyacrylamide gel.

differ from the interaction between B. bacteriovorus BdDnaA and the BdoriC. In contrast to B. bacteriovorus, in which only a single nucleoprotein complex was formed (Figure 1B), the interaction of E. coli and P. putida DnaAs with the BdoriC region yielded multiple discrete nucleoprotein complexes that formed a ladder of retarded bands on the gel indicating that the DnaA boxes were sequentially bound by the DnaA proteins (Figure 3). This difference in orisome formation prompted us to question how the arrangement of DnaA boxes and/or the properties of DnaA influence the formation of nucleoprotein complexes, and whether the formation of a single BdDnaA-BdoriC complex is specific to B. bacteriovorus. To answer these interesting questions, we analyzed orisome formation in heterologous systems. The BdoriC region from B. bacteriovorus was incubated with DnaA proteins from prey organisms (E. coli or P. putida) and the formed nucleoprotein complexes were analyzed using EMSAs. In these in vitro heterologous systems (as in the homologous system; Figure 1B), the observed nucleoprotein complexes formed in a protein-concentration-dependent manner (Figure 3). Surprisingly, the DnaA proteins of the prey organisms exhibited higher affinities toward the B. bacteriovorus oriC than the BdDnaA from B. bacteriovorus toward its own BdoriC region. The BdoriC region from B. bacteriovorus was almost completely bound at the lowest tested concentration of DnaA from E. coli and P. putida (1 nM; Figure 3), whereas in the homologous system, the nucleoprotein complex was detectable only at B. bacteriovorus.
DnaA concentrations > 3 nM (Figure 1B). As the concentrations of *E. coli* or *P. putida* DnaA proteins increased, the complexity of the band pattern increased until a critical point was reached, whereupon the ladder pattern was replaced by diffuse, highly retarded bands indicative of large complexes (Figure 3). Thus, whereas the *B. bacteriovorus* DnaA appears to exhibit a unique binding mode to the *B. bacteriovorus* oriC, the DnaA proteins of prey organisms bind their own and *B. bacteriovorus* oriC regions in a similar manner. We also observed that the oriC region from *B. bacteriovorus* was not specifically bound by DnaA proteins from non-prey organisms (e.g., *Streptomyces coelicolor*; data not shown).

Together, the results of in vitro analysis indicate that DnaA proteins from prey bacteria bind the BdoriC region specifically and with a high affinity, forming multiple nucleoprotein complexes.
DNA Unwinding Takes Place at the 5′-end of BdoriC

To experimentally verify the in silico-predicted *B. bacteriovorus* DUE within BdoriC, we used a P1 nuclease assay. Supercoiled pOCBdoriC plasmids containing all of the predicted DnaA boxes (Supplementary Table S1) were incubated with increasing amounts of BdDnaA and subsequently treated with P1 nuclease, which hydrolyzes single-stranded DNA at the opened helix and hence linearizes the unwound plasmid (Donczew et al., 2012). Subsequent digestion with SspI allowed us to approximate the region unwound by BdDnaA. We detected faint bands providing evidence for DnaA-dependent DNA unwinding; the observed DNA fragments were ~1.2 and 1.5 kb, indicating that the P1 hydrolysis site corresponded to the in silico-predicted DUE of *B. bacteriovorus* (Figure 4B1). We also observed an additional DNA fragment of about 2.3 kb (Figure 4A), likely corresponding to a DnaA-independent P1-sensitive site located within the plasmid origin of pBR322 (pOCBdoriC). It was previously suggested that the ori of pBR322 contains a helically unstable region (Kowalski et al., 1988; Donczew et al., 2012).

Since EcDnaA and PpDnaA strongly bound BdoriC, we examined whether these prey proteins could unwind BdoriC. Surprisingly, P1- and SspI-mediated digestion generated the same patterns obtained using BdDnaA (Figure 4B2,3), indicating that both proteins could in vitro unwind DNA within the BdoriC region. The efficiency of DNA unwinding was much higher for the prey proteins (particularly that of *E. coli*) than for BdDnaA (Figure 4B2,3). Moreover, when we performed P1 assays with an equimolar mixture of DnaA proteins from *B. bacteriovorus* and *E. coli*, strong bands were observed at a minimal protein concentration (35 nM; Figure 4B4) that was even lower than that found to yield a similar result with the *E. coli* protein alone (70 nM; Figure 4B2).

To precisely map the unwound region, we performed PE of P1-cleaved pOCBdoriC plasmids using Taq polymerase and 32P-labeled primers flanking the in silico-predicted DUE (for details see Materials and Methods and Supplementary Table S1). Since BdDnaA alone yielded only faint signals in the P1 assay, we used an equimolar mixture of DnaA proteins from *B. bacteriovorus* and *E. coli*, strong bands were observed at a minimal protein concentration (35 nM; Figure 4B2). The observed extension products confirmed that DNA unwinding occurs within the in silico-predicted DUE sequence at the 5′-end of the oriC region, and allowed us to estimate the unwound region as spanning ~55 bp (Figures 4C and 6A). Moreover, RIP mapping showed that BdoriC is the replication initiation site in vivo (Figure 5).

Together, these results show that BdoriC is unwound at the 5′-end by its own BdDnaA, as well as by DnaA proteins from the prey species, *E. coli* and *P. putida*.

BdoriC Is Not Able to Initiate DNA Replication in *E. coli*

Since BdoriC was specifically bound and unwound by the DnaA from *E. coli*, we next tested whether the predator’s oriC could initiate replication in its prey. To investigate ability of BdoriC to promote replication in *E. coli*, we performed a set of heterologous transformations in which the pBR322 plasmid (a negative control) and its derivatives carrying BdoriC or EcoriC (a positive control) regions (Supplementary Table S1) were assayed for oriC-dependent initiation of replication in the *E. coli* polA− strain (Langer et al., 1996; Zawilak-Pawlik et al., 2005). ColE1-type plasmids (such as pBR322) require DNA polymerase I for their replication; thus, only a construct containing a functional oriC

![FIGURE 5 | The replication initiation point is located within the BdoriC DUE.](image-url)
region (and conferring pBR322-encoded ampicillin resistance, AmpR) can replicate in the absence of DNA polymerase I (such as found in E. coli polA−). Among the analyzed constructs, only the pOC170 plasmid carrying the EcoRI C region was replicative (Table 1), suggesting that the BdoriC region does not promote the initiation of replication in E. coli. Indeed, no plasmid containing the BdoriC region yielded ampicillin-resistant transformants in the polA-deficient strain, even after prolonged incubation.

Together, these results show that B. bacteriovorus oriC is not a substrate for replication in its prey organisms.

DISCUSSION

Bdellovibrio bacteriovorus is a small predatory bacterium that exhibits a peculiar biphasic life cycle during which two different types of cells are produced: non-replicating highly motile cells (the free-living phase) and replicating cells (the intracellular-growth phase) (Starr, 1975). The process of chromosomal replication in B. bacteriovorus must therefore be temporally and spatially regulated to ensure that it is coordinated with cell differentiation and cell cycle progression. Although, we know that chromosomal replication in bacteria is mainly regulated at the initiation step, nothing is known about this process in B. bacteriovorus. Here, we report the first characterization of key elements of replication initiation, namely the BdDnaA protein and the BdoriC region, in a bacterium that preys on other bacteria. Surprisingly, we show that DnaA proteins from prey bacteria specifically bind and unwind the oriC region of their predator.

We identified the B. bacteriovorus oriC region within the rnpA-rpmH-dnaA-dnaN-recF-gyrB-gyrA gene cluster, which is conserved even in distantly related bacterial species (Ogasawara and Yoshikawa, 1992). BdoriC is localized between the dnaA and

| TABLE 1 | Replication activity of the BdoriC region in Escherichia coli. |
|----------------|-----------------------------|
|               | Transformation efficiency in E. coli (number of transformants per µg of DNA) |
|               | WM 1838 polA− (30°C) | WM 1785 polA+ (37°C) |
| Plasmid       |                             |                           |
| pBR322_BdoriC | 0                           | 4.79 × 10⁴                 |
| pOC170 (EcoRI) | 8.6 × 10⁴                 | 9.7 × 10⁴                 |
| pBR322        | 0                           | 12.8 × 10⁴                |
| pOCBdoriCΔEcoRI | 0                         | 8.8 × 10⁴                |
|               | 0                           | 7.6 × 10⁴                |

FIGURE 6 | Organization of the B. bacteriovorus oriC region. (A) The results from our in silico, in vitro and in vivo analyses. (B) Consensus sequence of the B. bacteriovorus DnaA box, as identified using an online tool WebLogo (Crooks et al., 2004).
**FIGURE 7** | The structures of the oriC regions from *B. bacteriovorus* and two of its prey bacteria. Underlined DUE indicates experimentally unconfirmed unwinding. The direction of each triangle represents the orientation of a DnaA box. The small arrows below gene names indicate their gene orientations.

DNA sequences are critical for the initiation of chromosomal replication. The oriC region consists of two main functional modules: the DUE region, responsible for DNA unwinding, and the DnaA-binding sites, which interact with the DnaA protein to initiate replication. In *B. bacteriovorus*, the oriC region is located near the dnaA gene, and its structure is similar to that of other bacteria, including *Escherichia coli* and *Pseudomonas putida*. The DUE region is located proximal to the first DnaA box, and its structure is conserved across various bacterial species, suggesting a common evolutionary origin.

Sequence comparison of the DnaA-binding sites enabled us to propose a consensus sequence for the *B. bacteriovorus* DnaA box, which is similar to the "perfect" box sequence found in *E. coli*. This suggests that the interaction of DnaA with these residues follows similar specificity rules in all of the tested bacterial species. Our findings are consistent with previous reports showing that these bases of the DnaA box are important for interactions between DnaA and the DnaB box. On the other hand, the first three positions in the DnaA box sequence appear to be relatively relaxed, and are thus likely to confer the species specificity of DnaA-DNA interactions.

The second basic functional module of the replication origin is the DUE region, which is responsible for the unwinding of DNA. In *B. bacteriovorus*, the DUE region is located near the dnaN gene. Our analysis revealed that the DUE region is AT-rich, but does not contain the typical AT-rich 13mer repeat found in other bacteria. However, we identified an array of four DnaA-trio elements (GAT) within the DUE region, which are conserved in phylogenetically distant organisms. This suggests that the interaction of DnaA with these residues follows similar specificity rules in all of the tested bacterial species. Our findings are consistent with previous reports showing that these bases of the DnaA box are important for interactions between DnaA and the DnaB box. Although the *B. bacteriovorus* DUE region is AT-rich, it does not contain the typical AT-rich 13mer repeat found in other bacteria (e.g., *E. coli* or *Bacillus subtilis*). However, we identified an array of four DnaA-trio elements (GAT) within the DUE region; they are
located near DnaA box 1, and are separated from this box by a short GC-rich region (Figure 6A). DnaA-trios are newly identified elements found within the DUEs of bacterial oriCs (Richardson et al., 2016). Recently, Richardson et al. (2016) demonstrated that these trios play an essential role in replication initiation by enabling DnaA to form a filament on single-stranded DNA, thereby promoting the unwinding of oriC.

The AT-rich sequences of the DUEs showed very little homology between B. bacteriovorus and E. coli or P. putida. Surprisingly, however, the DnaAs from these prey bacteria were found to unwind BdoriC within the AT-rich region in vitro (Figure 4B4). Moreover, compared to BdDnaA, EcDnaA, and PpDnaA were more efficient in opening double-stranded DNA at the DUE region of BdoriC. This is particularly interesting in the case of EcDnaA, which requires HU proteins to open the E. coli replication origin in vitro (Dixon and Kornberg, 1984; Hwang and Kornberg, 1992). Our in vitro experiments revealed that both EcDnaA and PpDnaA can unwind BdoriC in the absence of HU or any other ‘prey-derived’ protein (Figure 4B). This presumably indicates that DnaA proteins are intrinsically capable of unwinding a DUE once a proper DnaA oligomer has been formed. Such oligomerization depends on both the DnaA-box scaffold and the presence of additional regulatory proteins that help DnaA initiate complex formation (e.g., HU and DiaA in E. coli) (Hwang and Kornberg, 1992; Ishida et al., 2004; Keyamura et al., 2007). The factors responsible for stimulating this unwinding of DNA in B. bacteriovorus remain to be identified.

Interestingly, the in vitro binding mode of BdDnaA differs from those of EcDnaA and PpDnaA, despite having similar oriC regions structures (Figure 7). The binding of BdDnaA to BdoriC results in the formation of a single nucleoprotein complex (Figure 1B), whereas the binding of EcDnaA or PpDnaA to BdoriC yielded multiple discrete nucleoprotein complexes (Figure 3). This suggests that BdDnaA binds simultaneously at all eight boxes, whereas the DnaA proteins of the prey species sequentially bind the DnaA boxes within BdoriC. Moreover, EcDnaA and PpDnaA exhibited higher affinities toward BdoriC compared to BdDnaA. This further suggests that additional factors contribute to BdDnaA oligomerization/DNA binding, and thus may be involved in regulating the chromosomal replication of B. bacteriovorus.

It is noteworthy that the in vitro ability of prey’s DnaA proteins to bind and unwind BdoriC region may not necessarily reflect the in vivo situation. Growth of B. bacteriovorus in the periplasma of host bacteria and the production of many proteases represent some of limitations of transferring in vitro results to in vivo conditions.

Similar to another Gram-negative bacterium, Caulobacter crescentus, B. bacteriovorus exhibits a dimorphic life cycle in which replicative cells originate from non-replicative cells (Janakiraman and Bum, 2000). In C. crescentus, the master regulator, CtrA, temporally and spatially coordinates chromosomal replication with the developmental program by regulating the activity of oriC (Laub et al., 2002). In future work, we plan to identify one or more proteins that might control the initiation of chromosomal replication in B. bacteriovorus.

In sum, we herein identify the key elements of B. bacteriovorus chromosomal replication initiation, DnaA and oriC, and characterize their interaction in vivo and in vitro. We also show that DnaA proteins from prey bacteria bind the BdoriC region specifically and with high affinity, forming multiple nucleoprotein complexes. Finally, we demonstrate that BdoriC is unwound at 5’-end by its own DnaA as well as by those of the prey species, E. coli and P. putida. This work provides important new entry points toward improving our understanding of the initiation of chromosomal replication in this predatory bacterium.

AUTHOR CONTRIBUTIONS

LM, RD, and JZ-C designed research, LM performed in vitro and in vivo research, CW performed in silico research, AZ-P, RD, and CW performed critical revision, LM and JZ-C wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01898/full#supplementary-material

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**Conflict of Interest Statement:** 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.

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