Purification and Properties of the Plasmid Maintenance Proteins from the *Borrelia burgdorferi* Linear Plasmid lp17\(^7\)

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The Lyme disease spirochete *Borrelia burgdorferi* carries more plasmids than any other bacterium, many of which are linear with covalently closed hairpin ends. These plasmids have also been referred to as mini-chromosomes and essential genetic elements and are integral components of its segmented genome. We have investigated two plasmid maintenance proteins, BBD14 (the replication initiator) and BBD21 (a presumptive ParA orthologue), encoded by the linear plasmid lp17; these proteins are representatives of paralogous families 62 and 32, respectively. We have purified recombinant 6-his-BBD21 and shown it possesses an ATPase activity. 6-his-BBD14 initially could not be overexpressed in *Escherichia coli* by itself. It was only effectively overproduced in recombinant form through coexpression with other *B. burgdorferi* proteins and codon optimization. Although the mechanism for increased production through coexpression is not clear, this method holds promise for expression and purification of other *B. burgdorferi* proteins, a number of which have remained recalcitrant to purification from *E. coli*. Finally, we present evidence for the physical interaction of BBD14 and BBD21, a feature suggesting that BBD21 and the paralogous family 32 proteins are more likely involved in DNA replication than functioning as simple ParA orthologues as previously surmised based upon sequence homology. Such a role would not preclude a function in plasmid partitioning through interaction with the replication initiator.

The spirochete *Borrelia burgdorferi* and related *Borrelia* species are the causative agents of Lyme disease, a debilitating illness that has been reported by the U.S. Center for Disease Control and Prevention to be the fastest growing zoonotic illness that has been reported by the U.S. Center for Disease Control and Prevention. *B. burgdorferi* is highly segmented (8, 17) and contains a number of circular and linear plasmids, also referred to as mini-chromosomes (3) and essential genetic elements (42). The linear plasmids as well as the linear chromosome are also unusual in that they are terminated by covalently closed hairpin ends (2, 9, 24) whose generation by the telomere resolvase ResT has been characterized in depth (1, 13, 28–30, 45–47). However, the mechanisms involved in the peaceful coexistence of about two dozen DNA molecules remain unknown. Faithful replication and partitioning of these plasmids is of crucial importance to the organism, as a number of essential genes are carried by these extrachromosomal elements (7, 26, 27, 31, 36, 37, 39, 42, 50).

Our knowledge of the processes involved in plasmid maintenance in *Borrelia* species is rudimentary, at best. It is known that most of the plasmids of *B. burgdorferi* each carry between two and four members of five paralogous gene families (Fig. 1) believed to be involved in plasmid maintenance (8). The region encoding the clustered paralogous family (PF) members from several linear and circular plasmids has been shown to be sufficient to confer autonomous replication ability and therefore likely carries both the plasmid origins and the trans-acting factors involved (4, 15, 43, 44). Expression levels of some of these proteins are also correlated with plasmid copy number (5).

Every *B. burgdorferi* plasmid carries a member of either PF57 or PF62, two families that display low but significant similarity (8). Because of the universal presence of one of these related families on every plasmid, they are the most likely candidates for replication initiator proteins. PF57 is more commonly encountered and is found on 17 of the 22 plasmids. Some plasmids carry two apparently intact copies of the putative replication initiators and several plasmids carry initiator pseudogenes; these are the result of genome rearrangements in the linear plasmids (8) that are believed to occur via reversal of the telomere resolution reaction that generates the covalently closed hairpin ends on the linear plasmids (9, 29). No functional motifs have been identified in the PF57 and PF62 families, which only display significant similarity to proteins present on plasmids in other *Borrelia* species (22, 23).

It is known that replication of the linear *B. burgdorferi* chromosome starts from the center of the chromosome and proceeds bidirectionally toward the hairpin ends (35). Studies on the linear plasmid lp17 have shown that the region essential for replication is a 1.8-kb stretch from the center of the plasmid and that expression of BBD14, the PF62 member, is required (4). The origin must reside somewhere within this 1.8-kb region, but the precise location remains to be established. lp17 also carries a second putative plasmid maintenance gene, bbd21. This gene encodes a paralogous PF32 member (BBD21) with ~25% sequence conservation with a variety of members of the ParA family of partitioning proteins (8, 51). Bacterial partitioning systems come in two types: those that encode actin-like ATPases and those that encode Walker box...
ATPases (14). The latter type includes the ParA family of partitioning systems, which have three components: two trans-acting proteins and one cis-acting DNA site (20, 21). The centromere-like site (parS) is recognized by the ParB protein. The ParA ATPase then interacts with the ParB-parS complex to promote partitioning. The Walker A and Walker B boxes found in ParA members (19) are shown in Fig. 2. BBD21 lacks the N-terminal extension found in some ParA orthologues. All B. burgdorferi plasmids, with the exception of cp9 and lp5, carry a PF32 member; however, none of the plasmids encodes a ParB orthologue. Removal of the bbd21 gene from lp17 does not seriously affect plasmid maintenance (4); however, complementation by a PF32 member from a different plasmid cannot be ruled out, and the function of the PF32 members remains unknown.

Purification and characterization of B. burgdorferi plasmid maintenance proteins have not been previously reported. In this study we have overcome the inability for BBD14 overexpression through coupled codon optimization and coexpression. We have purified 6-his-BBD14 and 6-his-BBD21 and report an ATPase activity for 6-his-BBD21. We also present evidence for a physical interaction between BBD14 and BBD21, suggesting that they may function together in plasmid replication.

FIG. 1. Putative B. burgdorferi plasmid replication proteins. The 12 linear and 10 circular plasmids of Borrelia burgdorferi strain B31 are shown (8, 17, 32). The five paralogous gene families encoding plasmid maintenance proteins are denoted as colored circles (8). The four lp28 plasmids have been grouped by a bracket. This figure was adapted from reference 8 with permission of the publisher.

FIG. 2. Alignment of BBD21 with two related protein families. The proposed ATPase domain of four members of the ParA family and four members of the cobyrinic acid synthase family found through BLAST searches were aligned with BBD21 using ClustalW (11), followed by manual adjustment of the sequences. Completely conserved residues are shaded in orange, and similar residues (as defined by the Blossom62 matrix [25]) are yellow. The threshold for shading was 67% identity/similarity. Known ParA/ATPase motifs (19) are indicated above the sequence. Numbering on the bottom of the alignment corresponds to that of BBD21. White-on-blue residues are those that were mutated in this study. Similarity extends throughout the length of the proteins but is not shown here. The GenBank accession numbers of the aligned proteins are as follows: Soj protein in Rickettsia prowazekii, NP_220452; Spo0A activation inhibitor in Clostridium perfringens, NP_563568; MinD family ATPase, SOJ in Clostridium, NP_350310; soj protein in Rickettsia conorii, NP_359723; cobyrinic acid synthase of Desulfovibrio vulgaris, YP_965488.1; hypothetical protein RcanM of Rickettsia canadensis, ZP_01347170.1; cobyrinic acid synthase of Methylophilales bacterium sp. strain HTCC2181, ZP_01551553.1; cobyrinic acid synthase of Acidobacteriabacterium ellin, ABF39046.1.
argF

pJD164 with NdeI and HindIII. The ligation mixture contained 70 fmol of vector, description in Table 1. These plasmids were constructed by digesting either pET15b. The PCR conditions were as previously described (10). The plasmids

Bacterial strains and plasmids. BRL DH5α [F- (de8ΔInc2ΔMAL) Δ(lacZYA-

argF)U169 recA1 endA1 hsdR17 (rK-mK- mB-) gal dcm lacY1](DE3)pLySsRARE [F- ompT hsdSΔ(mk- mB-) gal dcm lacY1 (DE3)pLySsRARE (Cm')]) was used for the overexpression of mutant and wild-type BDB2 proteins. The recombinant plasmids used in this study are described in Table 1. When appropriate, antibiotics were added to the following concentrations: ampicillin (sodium salt; 100 μg/ml) or chloramphenicol (sodium salt; 30 μg/ml).

Construction of plasmids. By using PCR-based mutagenesis as described previously (49), with synthetic oligonucleotides (Table 2) and the 6-his-BDB2 overproducer pCB55 as a template, a number of plasmids carrying the bdb21 gene with a single amino acid substitution were constructed (pJD182 to pJD194)

TABLE 1. Plasmids used in this study

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| Plasmid      | GCE no. | Description | Reference or source |
|--------------|---------|-------------|---------------------|
| pET15b      | 523     | Expression vector, pBR322 replication origin, P<sub>lac</sub>, lacI, Ap<sup>R</sup> | Novagen             |
| pBBD14      | 805     | +Bluescript containing synthetic bdbb14 with optimal codon usage for E. coli, Km<sup>R</sup> | This work           |
| pCB55       | 486     | 6-his-BDB21, bdb21 in pET15b, pET15 Δ(Ndel-BamHI, 10 bp) | This work           |
| pJD212      | 360     | 6-His-ResT, resT<sup>I</sup> in pET15b, resT without internal Ndel sites, derived from pTB10 | 47                  |
| pJD125      | 706     | 6-his-BDB21, bdb21<sup>4</sup> original gene in pET15b, pET15 Δ[Ndel-BamHI, 10 bp] | This work           |
| pJD164      | 804, 806, 807 | 6-his-BDB21, bdb21<sup>4</sup> synthetic gene in pET15b, pET15 Δ[Ndel-BamHI, 10 bp] | This work           |
| pJD165      | 810, 811 | BBD21, no His tag, pCB55 Δ[Ncol-Ndel, 65 bp] | This work           |
| pJD219      | 1414, 1415 | 6-his-BBB21, bbb21<sup>4</sup> in pET15b, pET15 Δ[Ndel-BamHI, 10 bp] | This work           |
| pJD221      | 1419 | 6-his-BBB21, bbb21<sup>4</sup> in pET15b, pET15 Δ[Ndel-BamHI, 741 bp] | This work           |
| pJD222      | 1420 | BBD21<sup>2</sup>, no His tag, pJD164 Δ[Ndel-BamHI, 741 bp] | This work           |
| pJD229      | 1432 | 6-His-OspC, bbb19<sup>4</sup> in pET15b, pET15 Δ[Ndel-BamHI, 10 bp] | This work           |
| pJD182      | 852, 854 | 6-his-BBB21, K15A, pCB55 A13413G, A13414C, B. burgdorferi bbd21 | This work           |
| pJD183      | 853, 855 | 6-his-BBB21, T6A, pCB55 A13416G, B. burgdorferi bbd21 | This work           |
| pJD190      | 865, 866 | 6-his-BBB21, G14A, pCB55 G13411C, B. burgdorferi bbd14 | This work           |
| pJD191      | 867, 868 | 6-his-BBB21, K15E, pCB55 A13413G, B. burgdorferi bbd21 | This work           |
| pJD193      | 871, 872 | 6-his-BBB21, D123H, pCB55 G13737C, T13739C | This work           |
| pJD194      | 873, 874, 875 | 6-his-BDB21<sup>1</sup>, bbd21<sup>4</sup> Δ[A13371-C14108], B. burgdorferi bdb21 | This work           |
| pJD204      | 890, 891 | 6-his-BBD21, bbb21<sup>4</sup> in pET15b, pET15 Δ[Ndel-BamHI, 10 bp] | This work           |
| pJD207      | 895, 896 | 6-his-BBD21-BBB21<sup>4</sup>, pCB55 Δ[BamHI-HindIII, 290 bp] | This work           |
| pJD216      | 1410, 1411 | 6-his-BBD21-6-his-BBB21<sup>4</sup>, pCB55 Δ[BamHI-HindIII, 290 bp] | This work           |
| pJD212      | 1421 | BBD21<sup>2</sup>-6-his-BBB21<sup>4</sup>, pCB55 Δ[BamHI-HindIII, 290 bp] | This work           |
| pJD227      | 1429 | Rest<sup>1</sup>-6-his-BBB21<sup>4</sup>, pJD221 Δ[BamHI-PstI, 1,069 bp] | This work           |
| pJD230      | 1433 | 6-His-OspC-6-his-BBB21<sup>4</sup>, pJD229 Δ[BamHI-HindIII, 290 bp] | This work           |

* Coordinates are according to reference 4.  
* Nomenclature is according to Novick et al. (33).  
* GCE numbers are the group internal references to strains containing the described plasmids. 

MATERIALS AND METHODS

Expression of 6-his-BDB21 and 6-his-BBB21. Expression of 6-his-BDB21 (and/or 6-his-BBB21) was induced by +DhaS in Rosetta cells (200 ml, in LB medium containing 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, and 1% [wt/vol] glucose). Batches of cells, in volumes ranging from 200 to 1,000 ml, were grown at 36°C and shaken at 250 rpm. At an optical density at 600 nm of 0.5, the culture was then centrifuged (3,000 × g, 15 min, 4°C) and subsequently suspended in 50 ml lysozyme buffer containing 1 mM NaCl, 50 mM HEPES (pH 7.6), 1 mM EDTA (pH 8.0), 0.5% [wt/vol] spermidine, 5.5% [wt/vol] sucrose, 0.2% [wt/vol] Zwittergent 3-16 (Sigma), and 0.6 mg/ml lysozyme. The cells were incubated 30 min at 0°C, then freeze-thawed three times to lyse them, followed by centrifugation for 1 hour at 100,000 g. and the supernatant fluid was applied to a Ni-nitrotriacetic acid (NTA) column (Qiagen). Purification under native conditions was achieved as per the manufacturer's instructions, except that 10% glycerol was added to all buffers. Attempts to remove a contaminating ATPase activity by DEAE-Sepharose, heparin-Sepharose, and hydroxylapatite column purification were unsuccessful. Contaminating ATPase activity was removed by glycerol gradient centrifugation (see below for conditions). Purified protein was stored at −80°C and retained its enzymatic activity for at least 8 months.
Glycerol gradient centrifugation. A glycerol gradient was prepared containing 25 mM HEPES-NaOH (pH 7.6), 250 mM NaCl, 10 mM MgCl₂, and 15% to 45% glycerol. The gradient was overlaid with 50 to 100 ml of 1.5 M NaCl, 25 mM sodium phosphate (pH 7.6), and 10% glycerol, followed by centrifugation at 100,000 rpm for 18 h at 12°C. After centrifugation the gradient was dripped from the bottom of the centrifuge tube by puncturing with a needle. Fractions of 7 l each, resulting in 14 or 15 fractions) were collected, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and checked for ATPase activity as described below.

ATPase assay. ATPase activity was determined by incubating 30 pmol 6-his-BBD21 in reaction mixtures containing 20 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 10 mM MgCl₂, 10% glycerol, 100 µg/ml bovine serum albumin, 0.1 mM ATP with 1 µCi of [γ-32P]ATP in a total volume of 20 µl. After incubation at 30°C for 60 min, an aliquot of the reaction mixture was directly spotted onto a polyethyleneimine thin-layer plate that was developed with 1 M HCOOH and 0.5 M LiCl. The plate was dried and exposed to a Cyclone phosphorimaging plate, which was read in a Cyclone PhosphorImager, and the resulting image file was quantified using ImageQuant software (version 5.2) to calculate the ratio of free phosphate to total ATP.

RESULTS

Purification and properties of 6-his-BBD21. Molecular cloning of bbd1 into the expression vector pET15b (an expression vector containing a His tag at the N-terminus; Novagen) resulted in plasmid pCB55. Upon IPTG induction of this plasmid in E. coli Rosetta, which overexpresses the tRNAs for several rare codons, a soluble protein of about 30 kDa was overproduced. This molecular mass was close to the expected size of 6-his-BBD21 (30,930 Da) (Fig. 3, lanes 2 and 3). The protein (lane 4) was extracted from the cells and purified on a Ni-NTA column as described in Materials and Methods. The yield of protein at this stage was about 5 mg per liter of culture. The peak elution fraction from the Ni-NTA column was subjected to glycerol gradient centrifugation (lane 5) to remove contaminating ATPase activity as will be discussed below. The sedimentation coefficient of 6-his-BBD21 (2.4 S₂₀,₅₀) in glycerol gradients was close to that expected for monomeric 6-his-BBD21 (2.1 S₂₀,₅₀), suggesting that BBD21 behaves as a monomer in solution (data not shown).

Purification of a member of the B. burgdorferi PF32 proteins

![FIG. 3. Induction and purification of 6-his-BBD21. A Coomassie blue-stained 15% SDS-polyacrylamide gel is shown. Lanes: 1 and 6, molecular mass markers; 2, 10 µl of whole-cell extract of uninduced Rosetta cells containing pCB55 for overexpression of 6-his-BBD21; 3, 10 µl of whole-cell extract of Rosetta cells containing pCB55, induced by 1 mM IPTG and grown for 45 min at 37°C; 4, 10 µg of 6-his-BBD21 after purification with a Ni-NTA column; 5, 10 µg of Ni-NTA-purified 6-his-BBD21 after subsequent purification by glycerol gradient centrifugation.](image)
has not been previously reported. Purified 6-his-BBD21, the PF32 protein from lp17, was shown to have ATPase activity as predicted by in silico analysis of the amino acid sequence, its similarity to the ParA family of proteins, and the presence of Walker A and B boxes (Fig. 2). The ATPase assay was carried out as described in Materials and Methods by first purifying the peak fractions from the Ni-NTA column on a glycerol gradient centrifugation as shown in panels A and B. The fraction with the highest concentration of 6-his-BBD21 protein was assayed for ATPase activity as described in Materials and Methods. ATPase activity per pmol of protein is shown relative to wild-type 6-his-BBD21.

To prove that the ATPase activity comigrating with 6-his-BBD21 in the glycerol gradient was indeed a property of 6-his-BBD21, a deletion mutant missing amino acids 1 to 19 (6-his-BBD21Δ19), corresponding to the predicted Walker A Box, was constructed (Fig. 2). A loss in the ATPase activity associated with 6-his-BBD21 was observed upon glycerol gradient sedimentation of 6-his-BBD21Δ19 (Fig. 4B). In addition, several point mutations were introduced into the Walker A and B motifs of 6-his-BBD21. Specifically, the residues G14, K15, and T16 in the Walker A box and D123 in the Walker B box were changed to either A, E, or H (Fig. 2) as previously reported for the Walker boxes of P1 ParA (19). All the mutant proteins purified through the glycerol gradient step had substantially reduced ATPase activity (Fig. 4C), though no single point mutation completely abolished the enzymatic activity, as reported for the P1 ParA protein (19).

The ATPase activity observed for 6-his-BBD21 was not robust, as observed for many Walker box ATPases, but instead displayed a low specific activity of 0.5 pmol ATP/min per pmol 6-his-BBD21, as previously noted for the P1 ParA protein (0.2 pmol ATP/min per pmol ParA [12]). In contrast to ParA proteins, incubation of 6-his-BBD21 with ATP did not result in protein dimerization or multimerization, and the ATPase activity was not influenced by the presence of DNA (data not shown).

Finally, DNA binding activity of 6-his-BBD21 was assayed. Some of the ParA family members are known to be transcriptionally autoregulatory and bind to their own promoter regions (16, 18, 19). DNA binding assays to detect binding of 6-his-BBD21 to its own promoter region or to the region of lp17 known to carry the origin of replication (4) by footprinting, electrophoretic mobility shifts, or nitrocellulose filter binding assays did not reveal any sequence-specific DNA binding. However, sequence-independent binding to single-stranded and double-stranded DNA was observed (data not shown).

**Overexpression of 6-his-BBD14 through combined codon optimization and coexpression with other B. burgdorferi proteins.** To obtain a better understanding of BBD14, the putative replication initiation protein of lp17 (4), we made a number of attempts to overproduce the protein. We were unable to express 6-his-BBD14 at detectable levels using T7 promoter-containing vectors in E. coli Rosetta (Novagen) or other E. coli strains, including those deficient in several proteases. Use of a phage induction system (CE6; Novagen) (48) or the yeast *Pichia pastoris* expression system (34) also did not result in detectable 6-his-BBD14 levels. Moreover, use of a synthetic version of the *bbd14* gene, optimized for *E. coli* codon usage, did not improve the situation, and levels of 6-his-BBD14 remained undetectable either in a crude extract (Table 3, row 1 and 2) or after Ni-NTA chromatography (data not shown).

In a final effort to overexpress 6-his-BBD14, we generated a plasmid construct which carried both *bbd21* and 6-his- *bbd14* in tandem, such that both genes were transcribed to produce a bicistronic mRNA. Since these two proteins are believed to be involved in lp17 plasmid maintenance, the possibility existed for a physical interaction between the proteins and the stabi-
The expression levels were estimated from a Coomassie blue-stained SDS-PAGE gel containing 20 μl from the most concentrated elution fraction from the Ni-NTA column and with 1 μg bovine serum albumin as a reference protein.

The expression of 6-his-BBD14 was found to consistently coelute from the column (Fig. 6A). The coelution of His-tagged BBD14 resulted in a dramatic overproduction of 6-his-BBD14 (Table 3, row 3). The analogous construct, but with the native 6-his-bbd14 gene rather than the codon-optimized synthetic gene, resulted in some overproduction but about 25-fold less than with the synthetic gene (Table 3, row 4). Therefore, neither the synthetic gene alone nor the native gene coexpressed with bbd21 resulted in high-level expression. However, the synthetic gene coupled with bbd21 coexpression resulted in a dramatic overproduction of 6-his-BBD14. Reversal of the gene order resulted in a slight reduction (~40%) in the level of 6-his-BBD14 expression (Table 3, row 5).

To determine whether high-level 6-his-BBD14 production was specific to coexpression with BBD21, we generated several constructs where 6-his-bbd14 was coupled with other genes. With bbb21, the gene encoding the family 32 ParA orthologue from lp36, high-level expression was maintained (Table 3, row 6). Similarly, the resT gene encoding the B. burgdorferi telomere resolvase also promoted abundant production of 6-his-BBD14 (Table 3, row 7). Finally, coupling of the ospC gene, which encodes the outer surface protein C, also mediated high-level production of 6-his-BBD14 (Table 3, row 8). The ability of four B. burgdorferi proteins, including an outer surface protein, to support high-level production of 6-his-BBD14 in E. coli suggests that stabilization of the protein in E. coli by direct physical interaction is not the mechanism involved in mediating the overproduction (see Discussion, below).

**Fig. 5. Construct for coexpression of BBD21 and 6-his-BBD14.** A map of the coexpression plasmid pJD204, which expresses both wild-type bbd21 and His-tagged bbd14 from the same inducible T7 promoter is shown. The construct is derived from the pET15b vector (see Materials and Methods and Table 1). The sequence of the cistronic linker connecting bbd21 and 6-his-bbd14 is shown in blue below the map. Only the BamHI and NdeI sites used in the cloning are shown. The construct carries a codon-optimized synthetic 6-his-bbd14 gene (see Materials and Methods).

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**TABLE 3. Stabilization of BBD14 expression in E. coli by coexpression with other proteins**

| Expressed protein | His6-tagged BBD14 concn (mg/liter of cells) | Plasmid used |
|-------------------|--------------------------------------------|--------------|
| 6-his-BBD14 (native gene) | <0.025 | pJD125 |
| 6-his-BBD14 (synthetic) | <0.025 | pJD164 |
| BBD21 → 6-his-BBD14 (synthetic) | 25 | pJD204 |
| BBD21 → 6-his-BBD14 (native) | 1 | pJD216 |
| 6-his-BBD14 (synthetic) → BBD21 | 15 | pJD207 |
| BBK21 → 6-his-BBD14 (synthetic) | 25 | pJD223 |
| ResT → 6-his-BBD14 (synthetic) | 20 | pJD227 |
| 6-his-OspC → 6-his-BBD14 | 20 | pJD230 |

*The expression levels were estimated from a Coomassie blue-stained SDS-PAGE gel containing 20 μl from the most concentrated elution fraction from the Ni-NTA column and with 1 μg bovine serum albumin as a reference protein.

*b Synthetic bbd14 gene optimized for codon usage in E. coli.
BBD21: a ParA orthologue or a replication protein? The roles of BBD21 and other members of B. burgdorferi paralogous family 32 have not been elucidated. Every the B. burgdorferi B31 plasmid, with the exception of cp9 and lp5, carries a gene encoding a PF32 family member (8). These proteins were noticed early on to have similarity to the ParA family of proteins involved in plasmid partitioning (51). However, this sequence conservation is somewhat enigmatic in that plasmid partition systems invariably contain both a ParA and a ParB orthologue (14, 20), and B. burgdorferi plasmids do not encode any ParB-related proteins. Genetic experiments have not been helpful in defining the role of BBD21. Deletion of bbd21 from lp17 did not give rise to any obvious replication or partitioning phenotype; however, interpretation of these results was not clear cut, as the possibility for interplasmidic complementation by other PF32 members could not be ruled out (4).

In the work reported here we demonstrate that purified 6-his-BBD21 is an ATPase with a specific activity similar to that reported for P1 ParA. However, unlike ParA family members, the ATPase was not influenced by DNA. Moreover, ATP did not promote protein dimerization or oligomerization as observed for ParA family members (6, 14, 20). Sequence-specific DNA binding of 6-his-BBD21 to its own promoter region as described for ParA proteins with N-terminal extensions (16, 18, 19) could not be detected. There is a stretch of seven 21-bp direct repeats (GATATAAATTATATAGT), directly upstream of the ATG start codon for the bbd21 gene, but we were unable to detect convincing binding to this region using an electrophoretic mobility shift assay or by DNase footprinting, under a variety of conditions, including in the presence of ATP or ADP. This was not surprising, as BBD21 lacks the N-terminal extension found on the ParA proteins with sequence-dependent binding activity. However, 6-his-BBD21 does exhibit non-sequence-specific DNA binding activity, which may be important for its function.

Finally, we have observed a physical interaction of BBD21 with the replication initiator protein 6-his-BBD14 during purification. This interaction was not observed with BBK21, the ParA orthologue from lp56. Further attempts to study this interaction by protein-protein cross-linking and glycerol gradient centrifugation were not successful, suggesting that the interaction may be weak and/or transient. Taken together, the data suggest to us that BBD21 is not a strict functional ParA family member and that it is more likely to play an as-yet-unknown role in the DNA replication process of lp17. Such a role would not preclude a function in plasmid partitioning through interaction with the replication initiator. Additional experiments, including an in vitro replication system for B. burgdorferi plasmids, will be required to elucidate the role of BBD21. The methods described here to purify the two lp17 plasmids believed to be involved in plasmid replication should be invaluable tools in establishing an in vitro replication system and in further studies on this process.

**DISCUSSION**

**BBD21: a ParA orthologue or a replication protein?** The roles of BBD21 and other members of B. burgdorferi paralogous family 32 have not been elucidated. The family BBD14 consists of proteins that are encoded by other PF32 members could stimulate 6-his-BBD14 overproduction. Finally, coexpression of 6-his-BBD14 with a noninteracting partner (BBK21) facilitated purification of 6-his-BBD14 by itself, as shown in Fig. 6C.

A mixture of purified 6-his-BBD14 and BBD21 was assayed for site-specific DNA binding to a DNA fragment 1.9 kb in size (lp17, 7986 to 9831) to which the origin of replication had been localized (4); however, no sequence-specific DNA binding activity was observed by electrophoretic mobility shift assays. 6-his-BBD14 also did not affect the ATPase activity of BBD21 (data not shown).

**Overproduction of 6-his-BBD14 through codon optimization and coexpression.** The putative replication initiator protein BBD14 from lp17 was found to be refractory to overproduction in E. coli, a property not uncommon for expression of many recombinant B. burgdorferi proteins that are encoded by a genome that is about 75% A+T (8, 17). Synthesis of a bbd14 gene with optimal E. coli codon usage was attempted to remedy the undetectable levels of expression of this gene, which contains 53 rare E. coli codons (www.doe-mbi.ucla.edu/~sumchan/caltor.html). However, a synthetic gene did not

A mixture of purified 6-his-BBD14 and BBD21 was assayed for site-specific DNA binding to a DNA fragment 1.9 kb in size (lp17, 7986 to 9831) to which the origin of replication had been localized (4); however, no sequence-specific DNA binding activity was observed by electrophoretic mobility shift assays. 6-his-BBD14 also did not affect the ATPase activity of BBD21 (data not shown).

**FIG. 6. Coelution of BBD21 from Ni-NTA with His-tagged BBD14.** (A) Wild-type BBD21 and His-tagged BBD14 were coexpressed from a single promoter on pJD204 (Fig. 5). A Coomassie blue-stained 15% SDS-polyacrylamide gel of the elution profile from Ni-NTA purification step (see Materials and Methods). (B) Wild-type BBD21 was expressed in the absence of His-tagged BBD14 from pJD164 (Table 1). A Coomassie blue-stained 15% SDS-polyacrylamide gel is shown as for panel A. The lane labeled FT contains 20 μl of the flowthrough applied to the Ni-NTA column, showing that BBD21 did not bind to the column in the absence of His-tagged BBD14. (C) Wild-type BBK21 (the family 32 parologue from lp36) and His-tagged BBD14 were coexpressed from a single promoter on pJD223 (Table 1). A Coomassie blue-stained 15% SDS-polyacrylamide gel of the elution profile from Ni-NTA is shown. M denotes the marker lane with purified BBK21.
result in detectable overexpression unless the gene was coexpressed with the bbd21 gene. Coupling of a His-tagged native bbd14 with a bbd14 gene gave a low level of 6-his-BBD14 expression, about 25-fold lower than coupling when the synthetic 6-his-bbd14 gene was used. Optimal expression, therefore, required the synthetic 6-his-bbd14 gene coupled with bbd21 and was most effective when bbd21 preceded 6-his-bbd14. Since the two proteins copurified and appeared to physically interact, our initial thoughts were that BBD21 stabilized 6-his-BBD14 through direct physical interaction to facilitate protein folding or inhibit proteolysis. However, further studies where we coupled the 6-his-bbd14 gene with genes encoding other proteins not believed to interact, including the outer surface OspC protein, also resulted in similar levels of 6-his-BBD14 production to those observed with coupling to bbd21. The mechanism by which expression of 6-his-BBD14 is promoted by coexpression with noninteracting proteins remains enigmatic and might occur by improving mRNA stability, translational efficiency, or by inhibiting degradation by cellular proteases, in either case by some as-yet-undeclared mechanism(s). Nonetheless, the ability to now purify 6-his-BBD14 will allow future advancement in studies of lp17 replication. Moreover, the coupled codon optimization and coexpression approach successfully used for BBD14 may offer hope for the study of other B. burgdorferi proteins that have defied purification from recombinant plasmids in E. coli.

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