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The TamB ortholog of *Borrelia burgdorferi* interacts with the β-barrel assembly machine (BAM) complex protein BamA

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Summary

Two outer membrane protein (OMP) transport systems in diderm bacteria assist in assembly and export of OMPs. These two systems are the β-barrel assembly machine (BAM) complex and the translocation and assembly module (TAM). The BAM complex consists of the OMP component BamA along with several outer membrane associated proteins. The TAM also consists of an OMP, designated TamA, and a single inner membrane (IM) protein, TamB. Together TamA and TamB aid in the secretion of virulence-associated OMPs. In this study we characterized the hypothetical protein BB0794 in *Borrelia burgdorferi*. BB0794 contains a conserved DUF490 domain, which is a motif found in all TamB proteins. All spirochetes lack a TamA ortholog, but computational and physicochemical characterization of BB0794 revealed it is a TamB ortholog. Interestingly, BB0794 was observed to interact with BamA and a BB0794 regulatable mutant displayed altered cellular morphology and antibiotic sensitivity. The observation that *B. burgdorferi* contains a TamB ortholog that interacts with BamA and is required for proper outer membrane biogenesis not only identifies a novel role for TamB-like proteins, but also may explain why most diderms harbor a TamB-like protein while only a select group encodes TamA.

Introduction

Borrelia burgdorferi, the spirochete that causes Lyme disease, is classified as a diderm and is similar to Gram-negative organisms in that it contains both an inner membrane (IM) and outer membrane (OM) bilayer (Holt, 1978; Hovind-Hougen, 1984; Barbour and Hayes, 1986). Given that *B. burgdorferi* is an extracellular pathogen, the OM and its constituents are the major interface between the host and pathogen during infection (Fikrig *et al.*, 1990; 1992; Schaible *et al.*, 1990; Gilmore *et al.*, 1996; Ornstein *et al.*, 2002; Bunikis *et al.*, 2008). The borrelial OM is unique in many ways. For instance, the bilayer is not only composed of phosphatidylcholine and phosphatidylglycerol but it also contains free cholesterol, cholesterol esters, and three glycolipids [6-*O*-acylated cholesteryl β-D-galactopyranoside (ACGal), cholesteryl β-D-galactopyranoside (CGal), and mono-α-galactosyl-diacylglycerol

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(MGalD)] (Belisle *et al.*, 1994; Radolf *et al.*, 1995; Hossain *et al.*, 2001; Ben-Menachem *et al.*, 2003; Schroder *et al.*, 2003; Stubs *et al.*, 2009). Interestingly, the two cholesterol containing glycolipids (ACGal and CGal) along with free cholesterol have recently been shown to form ordered microdomains in the borrelial OM similar to eukaryotic lipid rafts (LaRocca *et al.*, 2010; 2013; Toledo *et al.*, 2015). Another significant feature of the *B. burgdorferi* OM is that, unlike Gram-negative bacteria, it lacks lipopolysaccharide (LPS) on its surface (Takayama *et al.*, 1987). Instead, the borrelial surface is decorated with lipid-modified proteins (i.e., lipoproteins) that are anchored into the outer leaflet of the OM (Fraser *et al.*, 1997; Casjens *et al.*, 2000; Brooks *et al.*, 2006; Kenedy *et al.*, 2012). The *B. burgdorferi* OM is also distinctive in that it contains a paucity of integral, membrane-spanning outer membrane proteins (OMPs) as compared to enteric Gram-negative organisms (Cullen *et al.*, 2004). In fact, it has been shown that there are 10-fold fewer OMPs in *B. burgdorferi* than in the OM of *Escherichia coli* (Lugtenberg and van Alphen, 1983; Radolf *et al.*, 1994). While these many distinctive aspects of the *B. burgdorferi* surface have been defined over the years, it is still poorly understood how biogenesis of its unique OM occurs.

A major advance in the field of bacterial OM biogenesis occurred just over a decade ago when assembly and export of OMPs in *Neisseria meningitidis* was determined to be controlled by an essential and highly conserved OMP designated Omp85 (Voulhoux *et al.*, 2003). A similar finding by Silhavy and co-workers in *E. coli* identified a homolog of Omp85, designated YaeT, that was shown to be member of a large multi-protein complex required for the OM assembly process (Wu *et al.*, 2005). It has also been recognized that proteins structurally and functionally similar to Omp85 and YaeT exist not only in all diderm bacteria but they are found in double-membraned eukaryotic organelles as well, such as mitochondria and chloroplasts (Gentle *et al.*, 2004; Zeth, 2010; Schleiff *et al.*, 2011). The YaeT and Omp85 proteins along with their homologs in other diderm bacteria have since been designated BamA and the larger complex is referred to as the β-barrel assembly machine (BAM) (Kim *et al.*, 2007; Walther *et al.*, 2009; Hagan *et al.*, 2011). The BAM complex is required for proper folding, assembly and export of many OMPs as they move from the periplasm into the bacterial OM, which is crucial to OM biogenesis (Wu *et al.*, 2005; Malinverni *et al.*, 2006; Kim *et al.*, 2007; Hagan *et al.*, 2011; Lenhart and Akins, 2010; Dunn *et al.*, 2015). The *E. coli* BAM complex has been well-characterized and consists of five members, the central OMP BamA and four accessory lipoproteins termed BamB, C, D and E (Wu *et al.*, 2005; Onufryk *et al.*, 2005; Ruiz *et al.*, 2005; Malinverni *et al.*, 2006; Sklar *et al.*, 2007; Walther *et al.*, 2009). Previous studies by our laboratory have revealed that the BAM complex in *B. burgdorferi* is much more compact consisting only of BamA and two accessory lipoproteins, BamB and BamD (Lenhart and Akins, 2010; Lenhart *et al.*, 2012; Dunn *et al.*, 2015). The overall structure of BamA is comprised of a C-terminal transmembrane β-barrel domain that is embedded in the OM and a large N-terminal periplasmic component divided into five distinct polypeptide transport-associated (POTRA) domains (Sanchez-Pulido *et al.*, 2003; Gentle *et al.*, 2005; Gatzeva-Topalova *et al.*, 2008; Noinaj *et al.*, 2015). The POTRA domains are prerequisites for BamA interactions with nascent OMPs and the accessory proteins of the BAM complex (Kim *et al.*, 2007; Gatzeva-Topalova *et al.*, 2010; Anwari *et al.*, 2010; Heuck *et al.*, 2011; Workman *et al.*, 2012; Bakelar *et al.*, 2016).

In addition to BamA, several other bacterial OMPs have recently been identified that also belong to the Omp85 protein family, including the TamA, TamL, patatin-Omp85, WD40-Omp85, metallo-Omp85 and noNterm-Omp85 proteins (Selkrig *et al.*, 2012; Heinz and Lithgow, 2014; Heinz *et al.*, 2015). Among these other Omp85 family members TamA has been the best characterized (Gruss *et al.*, 2013; Selkrig *et al.*, 2012; 2015; Shen *et al.*, 2014). Similar to the structure of BamA, TamA also consists of a C-terminal OM-associated β-barrel region and an N-terminal periplasmic region that contains three POTRA domains (Gruss *et al.*, 2013). TamA was first identified in the *Enterobacteriaceae* by Lithgow and co-workers and was shown to be part of a distinct complex termed the translocation and assembly module (TAM) (Selkrig *et al.*, 2012). The TAM consists of TamA and TamB; the OMP TamA interacts with the IM protein TamB and together they facilitate the assembly, localization, and export of many proteins, including autotransporters and other β-barrel OMPs such as fimbrial usher proteins and intimin (Selkrig *et al.*, 2012; Heinz *et al.*, 2016; Stubenrauch *et al.*, 2016). Phylogenetic analyses have indicated that a possible gene duplication of BamA in a *Proteobacterial* ancestor resulted in the generation of TamA (Heinz *et al.*, 2015). Thus, TamA is found only in *Proteobacteria*. On the other hand, TamB is ubiquitously distributed throughout all diderms with only a few rare exceptions (Heinz *et al.*, 2015). This latter point suggests that TamB plays an important but yet-to-be-identified function other than its well-characterized role in the *Proteobacterial* TAM.

Previous studies have revealed that TamB is anchored to the IM by an uncleaved signal peptide at its N-terminus, while its C-terminal region contains a conserved domain of unknown function 490 (DUF490) (Selkrig *et al.*, 2012; 2015; Gallant *et al.*, 2008). Additionally, TamB orthologs are typically found to be encoded contiguous to or in close proximity to proteins from the Omp85 family (Heinz *et al.*, 2015). For example, TamB is found in an operon with TamA in the *Proteobacteria* (Heinz *et al.*, 2015; Gallant *et al.*, 2008; Milner *et al.*, 2014), downstream of TamL in *Bacteroidetes* spp. and *Chlorobi* spp. (Heinz *et al.*, 2015), and in close proximity to BamA in organisms from the early-branching phyla, such as the *Fusobacteria*, *Dictyoglomi* and *Firmicutes* (Heinz *et al.*, 2015). Interestingly, *B. burgdorferi* encodes a single protein, BB0794, containing a DUF490 motif and it is located immediately upstream of BamA. This observation led to the suggestion that BB0794 is a TamB ortholog, although there is currently no experimental proof supporting this conjecture (Selkrig *et al.*, 2012). In the present study, we performed a detailed physicochemical characterization of BB0794. Signal peptide predictions, secondary structure modeling, and cellular localization assays revealed that BB0794 is anchored to the borrelial IM by an uncleaved signal peptide, which is similar to other TamB proteins. However, unlike TamB from the *Proteobacteria*, BB0794 is apparently essential for *B. burgdorferi* viability. Thus, an IPTG-regulatable *bb0794* mutant was generated. Examination of the mutant revealed that BB0794 is required to maintain normal bacterial morphology in *B. burgdorferi*. Moreover, co-immunoprecipitation experiments indicated that BB0794 specifically interacts with BamA. The combined data provide strong support for our conclusion that BB0794 is the TamB ortholog. Finally, the interaction observed between BB0794 and BamA leads us to speculate that TamB orthologs play an important role in OM biogenesis beyond their currently described function in the *Proteobacterial* TAM (Selkrig *et al.*, 2012).

Results

Domain structure and genetic organization of bb0794

BB0794 was recently suggested to be a TamB homolog in *B. burgdorferi* (Selkrig *et al.*, 2012). Despite an overall lack of sequence similarity between BB0794 and TamB (11% identity and 25% similarity score) BB0794 does contain two features conserved among all TamB orthologs, an N-terminal signal peptide and a C-terminal 325 amino acid region containing a DUF490 domain (Fig. 1A) (Heinz *et al.*, 2015). The DUF490 domain of BB0794, however, is only 13% identical and 28% similar to the DUF490 domain from the *E. coli* TamB, but it is predicted to have a secondary structure similar to all other DUF490 members consisting of multiple β-strands separated by a region of α-helix (Fig. 1B). Additionally, TamB proteins in other organisms are typically encoded within an operon containing a member of the Omp85 family of proteins, such as TamA, TamL, or BamA (Heinz *et al.*, 2015). Upon examination of the genetic locus containing BB0794, we observed that the gene encoding BB0794 is found immediately upstream of the genes encoding BamA and the periplasmic chaperone Skp, which together could possibly constitute a three gene operon. The genetic loci comparison among the most widespread genospecies, *B. burgdorferi*, *B. garinii* and *B. afzelii* are shown in Fig. 1C.

Expression of BB0794 and analysis of its physicochemical nature

The *E. coli* TamB protein is constitutively expressed and located in the periplasm (Selkrig *et al.*, 2012; 2015; Shen *et al.*, 2014). Interestingly, the borrelial genome deposited in the NCBI database for *B. burgdorferi* B31 (NCBI accession AE000783) has a missing nucleotide in *bb0794* gene which is annotated as a ‘K’ at base position 2395. The comparable base is noted as a guanine (G) in the annotated *B. garinii* and *B. afzelii* genomes. Resequencing this region in *B. burgdorferi* revealed that base 2395 corresponds to a G nucleotide in *B. burgdorferi* and the entire *bb0794* sequence encodes a predicted protein of 1465 amino acids. This analysis of the *bb0794* sequence is also supported by a recent resequencing of the *B. burgdorferi* genome that also indicated this base corresponds to a G in the *bb0794* gene (NCBI accession CP009656). To verify the gene sequencing results, we subsequently analyzed BB0794 expression and performed immunoblot analysis of borrelial whole cell-lysates using BB0794-specific antibodies. As expected from the corrected sequence analysis, we observed a protein of the expected size (168 kDa) expressed by *B. burgdorferi*, *B. garinii* and *B. afzelii* (Fig 2A).

To determine the physicochemical properties of BB0794 we performed Triton X-114 phase partitioning (TX-114 PP) to first determine if it is expressed as a soluble periplasmic protein or it has amphiphilic character similar to membrane proteins. This assay can separate membrane proteins from periplasmic or cytoplasmic proteins based on their overall solubility in Triton X-114 (Bordier, 1981; Brusca and Radolf, 1994). Membrane proteins partition into the detergent-enriched phase while soluble proteins partition into the aqueous phase (Fig. 2B). When Triton X-114 separated fractions were subjected to immunoblot using BB0794-specific antibodies, BB0794 was found exclusively in the detergent-enriched phase (Fig. 2C), which indicated BB0794 is a membrane protein. Immunoblot analyses for the

membrane-associated lipoprotein BamB and the soluble periplasmic protein Skp also were included as controls for the detergent and aqueous phases respectively.

Localization of BB0794 to the IM

Since TX-114 PP indicated that BB0794 is a membrane-associated protein, we next examined the localization of BB0794 by surface immunofluorescence assays (IFA) using BB0794-specific antisera (Fig. 3A). This analysis revealed that BB0794 could only be visualized when the spirochetal OM had been permeabilized, which was similar to the staining pattern observed for the periplasmic protein FlaB. OspA antibodies were included as a control outer surface protein and, as expected, OspA could be observed on the surface of intact spirochetes.

Although the immunofluorescence results indicated that BB0794 is not surface-exposed, to rule out the possibility that a surface-exposed domain was not accessible to antibodies by IFA, we also performed proteinase K (PK) experiments using whole *B. burgdorferi* cells. Consistent with the IFA result, the PK experiment revealed that BB0794 was not degraded by PK treatment (Fig. 3B). By contrast, the known surface-exposed protein OspA was found to be completely degraded by PK while the IM lipoprotein OppAIV, as expected was not degraded. Finally, when membrane fractionation was performed on *B. burgdorferi* to separate OM and protoplasmic cylinder (PC) fractions, BB0794 was found to be associated only with the PC fraction (Fig. 3C). For the fractionation experiments, antibodies specific for BamA were used as a control for a known OMP. BamA was observed in both the OM and PC preparations, as expected. To ensure the OM fractions were highly enriched and devoid of PC contents, we confirmed that the IM lipoprotein OppAIV was identified only in the PC fraction. The combined localization studies indicate that BB0794 is not localized to the OM and is associated only with the borrelial PC fraction.

BB0794 is anchored to the IM by an uncleaved signal peptide

The TamB protein from *E. coli* has a hydrophobic stretch of approximately 25 amino acids at its N-terminus that anchors it to the IM (Selkrig *et al.*, 2012; Shen *et al.*, 2014). Interestingly, the first 39 amino acids of the BB0794 sequence also is comprised of a hydrophobic domain based on Kyte-Doolittle hydrophobicity scores (Kyte and Doolittle, 1982). The PrediSi signal peptide prediction program, however, predicted that the N-terminus of BB0794 corresponds to a Sec-dependent signal peptide that is cleaved by signal peptidase I at amino acid 34 (data not shown) (Nielsen *et al.*, 1997). A more detailed analysis of BB0794 using the transmembrane domain identification algorithms TMHMM and Phobius revealed that other than the N-terminal hydrophobic region corresponding to first 34 amino acids (Krogh *et al.*, 2001; Kall *et al.*, 2004) there were no other potential transmembrane domains in BB0794 (data not shown). Therefore, to determine if the N-terminal hydrophobic region of BB0794 is a cleaved or an uncleaved signal peptide we fused the first 39 amino acids of BB0794 to the mature OspA sequence lacking its native signal peptide, which was designated OspA^{794N}. The OspA^{794N} construct and a full-length OspA with its native signal peptide (OspA^{WT}) were then expressed in *B. burgdorferi* strain B313. Strain B313 was utilized for these experiments since it does not contain the liner plasmid 54 (lp54) element that harbors OspA in *B. burgdorferi* (Sadziene *et al.*, 1995). Subsequent

immunoblot analysis revealed that the OspA^{794N} chimera containing the BB0794 signal peptide migrated several kDa larger than OspA^{WT} protein containing its native, cleaved signal peptide (Fig. 4A). This observation strongly suggested that the N-terminal region of BB0794 corresponds to an uncleaved signal peptide. To provide further support for this contention, we subjected whole-cell lysates from strain B313 expressing OspA^{794N} to TX-114 PP. When the final Triton X-114 detergent-enriched and aqueous fractions were subjected to immunoblot with OspA antibodies, the OspA^{794N} chimera partitioned exclusively into the detergent phase, confirming that OspA^{794N} is a membrane-associated amphiphilic protein (Fig. 4B). However, the mature recombinant OspA, which was expressed without its signal peptide (rOspA^{mat}), lacks the N-terminal lipidation site and therefore separates solely into the aqueous phase as a soluble protein after Triton X-114 PP (Fig. 4B). The membrane bound lipoprotein BamB and the hydrophilic protein Skp were used as whole cell-lysate controls to ensure full separation of the detergent and aqueous phases respectively. Finally, when PK surface accessibility assays were performed on OspA^{794N} it was not degraded by PK, although the surface-exposed OspA^{WT} protein was fully degraded by PK (Fig. 4C). As controls for the PK assays, the known PK sensitive protein P66 was shown to be partially degraded in the presence of PK while the IM-associated OppAIV was protected from PK degradation, as expected (Fig. 4C). The combined data indicated that BB0794 encodes an uncleaved signal peptide.

BB0794 interacts with BamA in *B. burgdorferi*

The DUF490 domain of TamB has been shown to specifically interact with the POTRA1 domain of TamA (Selkrig *et al.*, 2015). A TamA ortholog, however, is not present in *B. burgdorferi* and the only POTRA domain-containing protein in *B. burgdorferi* is BamA (BB0795), which is encoded just downstream of BB0794. To examine the possibility that BB0794 might interact with the POTRA containing BamA protein in *B. burgdorferi* we next performed co-immunoprecipitation (co-IP) experiments using anti-BB0794 antibodies. This analysis revealed not only that BB0794 antibodies can co-immunoprecipitate BB0794 along with BamA but that BamA-specific antibodies can specifically co-immunoprecipitate BB0794 (Fig. 5). Antibodies against OppAIV were included in this experiment to confirm that the interaction between BB0794 and BamA was specific. GST antibodies also were used to ensure the BB0794/BamA interactions observed were not the result of nonspecific antibody binding.

Generation of a regulatable BB0794 mutant strain

Prior studies on TamB have shown that its expression is not essential for bacterial viability (Gallant *et al.*, 2008; Selkrig *et al.*, 2012). After repeated attempts, however, we were unsuccessful in obtaining a *bb0794* deletion mutant in *B. burgdorferi*. To overcome this obstacle, we generated an IPTG-regulatable *bb0794* gene as shown in Fig. 6A. PCR analysis using specific primer pairs (white and black arrowheads in Fig. 6A) identified one mutant that had undergone recombination as expected (Fig. 6B). Since *B. burgdorferi* contains a large number of extrachromosomal linear and circular plasmids (cp), we also assessed the plasmid content of the mutant strain, designated *flacp::bb0794*. An analysis of all 21 endogenous plasmids in the parental and mutant strain revealed that the mutant maintained all plasmids except lp21 and cp32-7, neither of which have been implicated in borrelial

physiology, virulence or the overall life-cycle of this organism in the tick or mammal (Purser and Norris, 2000; Stevenson *et al.*, 2000; Caimano *et al.*, 2000; Terekhova *et al.*, 2006; Casjens *et al.*, 2012). As expected, the expression of BB0794 in the *flacp::bb0794* mutant was significantly reduced when IPTG was not included in the growth medium, although we never saw complete inhibition of BB0794 expression indicating that the regulation system is not fully repressed even when IPTG is absent. When IPTG was added, as little as 0.05 mM IPTG resulted in expression of BB0794 to a level similar to that observed with addition of 1 mM IPTG or in the parental strain (Fig. 6C). Interestingly, the expression of both BamA and Skp did not differ with the varying levels of IPTG in culture (Fig. 6C) even though it appeared that BB0794/BamA/Skp were part of a single operon and we assumed the BB0794 promoter would drive expression of these two downstream genes. As controls for the expression analyses, the level of the LacI repressor was also examined to ensure that it was expressed similarly in both the parental strain and in the mutant strain using differing levels of IPTG, and OppAIV was included to confirm that equal amounts of whole-cell lysates were loaded for all samples examined (Fig. 6C).

The observation that BB0794 expression was regulated by IPTG while BamA and Skp were not, indicated that these genes are not part of a single operon. To examine this issue, RNA-sequencing transcriptome data were analyzed to identify the 5′ ends of the mRNAs corresponding to *bb0794*, *bamA* and *skp*. As observed in previous deep-sequencing studies, sequencing reads accumulated with a pronounced peak at the 5′ end of each gene; this bias allows the relatively precise identification of transcription start sites (TSSs) based on the 5′ end of mapped reads (Perkins *et al.*, 2009; Hansen *et al.*, 2010; Raghavan *et al.*, 2011a, 2011b). This analysis revealed that BB0794 and BamA/Skp are encoded on distinct transcriptional units (Fig. 6D). The mRNA start site for *bb0794* was identified 13 bp upstream of the *bb0794* start codon and a single mRNA transcriptional start for both BamA and Skp was identified 343 bp upstream of the *bamA* start codon. The −10 and −35 promoter binding regions for *bb0794* and *bamA-skp* also were mapped by BPROM and virtual footprint v.3.0 (Munch *et al.*, 2005; V. Solovyev, 2011), which correlated with the TSSs identified (Fig. 6D). No transcriptional start was identified upstream of *skp*, which further indicated that *bamA* and *skp* are co-transcribed. To our knowledge, a TamB or TamB-like protein has not yet been reported to be essential for survival, which makes this the first study to suggest that the expression of BB0794/TamB-like protein is essential and further underscores the indispensable requirement of BB0794 in this spirochete.

BB0794 impacts normal *B. burgdorferi* morphology

We next examined the growth characteristics of the *flacp::bb0794* mutant with or without IPTG induction of BB0794 (Fig. 7A). While cell density between the parental and *flacp::bb0794* mutant strain cultivated with 1 or 0.05 mM IPTG was similar at all time points, the mutant cultivated without IPTG had significantly fewer organisms throughout the exponential and stationary phases of growth. Although the *flacp::bb0794* mutant was consistently observed to be at a lower cell density, the log phase doubling time of the mutant cultivated without IPTG (9.2 h) was similar to the parental strain (9 h) and the mutant cultivated with 0.05 or 1.0 mM IPTG (10 and 9.3 h respectively). During the growth analyses we observed that the *flacp::bb0794* mutant strain cultivated without IPTG exhibited

a distinct morphology with many of the spirochetes appearing to be long chains of unseparated organisms (Fig. 7B, left panel). While this phenotype can also account for the decreased total count of mutant spirochetes in growth assays, we did not observe any other alterations in bending, flexing, or overall motility of the extended length organisms. This notable and easily observable phenotypic alteration due to BB0794 depletion was fully rescued in the *flacp::bb0794* mutant cultivated in the presence of either 0.05 or 1 mM IPTG (Fig. 7B).

Deficiency of BB0794 increases the sensitivity of *B. burgdorferi* to antimicrobials

Previous studies have revealed that TamB plays an important role in maintaining OM integrity and morphology (Gallant *et al.*, 2008; Babu *et al.*, 2011; Selkrig *et al.*, 2012; Smith *et al.*, 2016). Given that we also observed the morphology of the BB0794 mutant to be altered, we next examined if the membrane integrity of the mutant also was impacted using antimicrobial sensitivity assays. We assessed the minimum inhibitory concentration (MIC) in the parental strain and the *flacp::bb0794* mutant cultivated with or without IPTG using a group of hydrophilic antibiotics (penicillin G, carbenicillin and cefotaxime) that typically enter the cell through porins and a group of hydrophobic antibiotics that preferably enter the cell by crossing through the membrane bilayer (tetracycline, minocycline, azithromycin). As shown in Table 1, the *flacp::bb0794* mutant cultivated in the absence of IPTG had a two-fold higher sensitivity to the hydrophobic antibiotic group (tetracycline, minocycline and azithromycin) as compared to the parent strain. The MIC values for the hydrophilic antibiotic group (penicillin, carbenicillin and cefotaxime) were not altered between the parental strain and mutant strain cultivated without IPTG. The combined data suggest that BB0794 plays a direct or indirect role in regulating and maintaining membrane permeability.

Discussion

TamB is a protein with a DUF490 domain that was recently shown to be encoded downstream of TamA (Selkrig *et al.*, 2012; 2015). TamA is an OMP and TamB is an IM-associated protein and together they interact in the periplasm to comprise the translocation and assembly module (TAM) (Selkrig *et al.*, 2012; 2015; Shen *et al.*, 2014). Similar to the BAM complex, the TAM from *Enterobacteriaceae* also catalyzes the insertion of OMPs into the OM of diderm bacteria (Selkrig *et al.*, 2012). Additionally, the TAM and BAM both contain a member of the Omp85 family of OMPs (TamA and BamA). Interestingly, while most *Proteobacterial* organisms contain the canonical TAM, many other bacterial phyla and even some *Proteobacteria* do not contain TamA or a TamA-like protein (Heinz *et al.*, 2015). By contrast, almost all diderm bacteria encode a TamB-like protein with a DUF490 domain found in close proximity or contiguous to an Omp85 family member such as TamA, TamL, or BamA (Heinz *et al.*, 2015). The *B. burgdorferi* BB0794 protein was found to be encoded upstream of BamA and to contain a C-terminal conserved DUF490 domain with predominance of a short β-sheet secondary structure. Cellular localization and membrane fractionation studies revealed it is an IM protein and proteinfusion assays confirmed that the BB0794 N-terminus is an uncleaved signal peptide. The combined data provide multiple lines of empirical evidence indicating that BB0794 should now be designated the *B. burgdorferi* TamB ortholog.

The region in the genome encoding BB0794/BamA/Skp is conserved not only in the Lyme disease spirochetes but among all spirochetes in the family *Borreliaceae*, which also includes the genera *Borrelia* that represents relapsing fever spirochetes. Within the *bb0794-bamA-skp* genetic locus in *B. burgdorferi*, we observed the presence of only a small intergenic 17 bp region between the *bb0794* and *bamA* genes and a 20 bp region between the *bamA* and *skp* genes, leading to the assumption that all three genes are part of a single transcriptional unit and constitute an operon. Examination of RNA sequencing data revealed two unique 5′ ends, one upstream of *bb0794* and another upstream of *bamA*, indicating that *bb0794* and *bamA* are separately regulated. While it is possible that the transcript encoding BB0794 could also encode BamA and/or Skp, immunoblot analysis of the IPTG-regulatable strain indicates otherwise since the BamA and Skp levels were observed to be independent of BB0794 expression levels. This leads us to conclude that BB0794 is primarily translated by the mRNA that starts upstream of *bb0794* while BamA and Skp are primarily translated from their own transcript. This raises the intriguing possibility that BB0794 and BamA/Skp expression needs to be independently regulated, which could mean that different environmental factors such as changes in temperature, host environment or physiological status of the organism requires differing levels of BB0794 and/or the BamA/Skp proteins. This notion is consistent with the finding that *bb0794* but not *bamA-skp* is regulated by the two-component system Hk1/Rrp1, which controls cyclic-di-GMP levels in *B. burgdorferi* (Rogers *et al.*, 2009; Caimano *et al.*, 2015). The recent study by Caimano and co-workers also indicates that *bb0794* transcription is up-regulated during the tick phase of the spirochetal enzootic cycle independent of *bamA* or *skp* expression (Caimano *et al.*, 2015), which would be consistent with BB0794 expression needing to be tightly regulated during the borrelial enzootic cycle while BamA and Skp can be constitutively expressed in this spirochete.

Given the role of the TAM in OMP export and secretion, it is not surprising that TamB mutants in *E. coli and Citrobacter rodentium* have been shown to have diminished translocation and secretion of the autotransporter proteins Ag43 and p1121, respectively (Selkrig *et al.*, 2012). Deletion of TamB also has been shown to increase serum sensitivity of *Salmonella enterica*, suggesting TamB helps to regulate OM composition in this organism at some level (Selkrig *et al.*, 2012). Furthermore, recent studies on MorC, a TamB ortholog from *Aggregatibacter actinomycetemcomitans*, have shown that this protein regulates the secretion of leukotoxin LtxA and also is required to maintain normal membrane morphology (Gallant *et al.*, 2008; Kachlany, 2010; Smith *et al.*, 2016). These combined studies suggest that TamB and TamB-like proteins in other organisms are not only involved in the proper export of various groups of OMPs, but they also are important for sustaining membrane integrity. Consistent with this observation, we also found that when BB0794 expression was depleted there were obvious defects in cellular morphology and in the overall sensitivity of *B. burgdorferi* to selected antibiotics, especially outer membrane traversing antibiotics. Along these lines, it is interesting to note that a mutant in SSG4 (substandard starch grain 4), which is a eukaryotic DUF490 domain-containing protein found in the amyloplasts of the rice plant *Oryza sativa* (Matsushima *et al.*, 2014), is observed to form enlarged starch granules due to defective septum formation in dividing plastids (Matsushima *et al.*, 2014). The *ssg4* mutant identified was found to contain a single mutation (Gly substitution for Ser)

in its DUF490 domain (Matsushima *et al.*, 2014). This specific Gly residue was found not only to be conserved in eukaryotic DUF490 domains but also among DUF490 domains of *Enterobacteriaceae* (Matsushima *et al.*, 2014). Interestingly, the conserved Gly residue also was identified in the DUF490 domain proteins of all Lyme disease causing *Borrelia* genospecies as well as in all relapsing fever *Borrelia* spp. and in *Treponemes* such as *T. pallidum* and *T. denticola* (data not shown). The role of this conserved DUF490 Gly residue found among not only pathogenic spirochetes but also in other diderms and eukaryotic organelles suggests it could be an important region to examine in future functional studies.

The most intriguing observation of the current study was the finding that BB0794 specifically interacts with BamA, indicating that TamB-like proteins form a previously unrecognized and distinct export system with the BAM complex. A possible explanation for the morphological defect observed in the BB0794 mutant could be the result of inhibiting this BB0794-BamA interaction. Since it has been established that the BAM complex is essential and required for OM biogenesis, it is tempting to speculate that the interaction between an inner and outer membrane-associated protein such as BB0794 and BamA is vital for coordinating the membrane syntheses and cell division process in this spirochete. Failure to meticulously coordinate these important physiological parameters could result in improper cell division, which was observed when BB0794 expression was depleted in the regulatable mutant.

Antibiotic sensitivity studies in *E. coli* have revealed that deletion of TamB results in increased sensitivity to vancomycin (Babu *et al.*, 2011). Additionally, MorC mutants in *A. actinomycetemcomitans* have significantly higher sensitivity to bile salts (Smith *et al.*, 2016). Similarly, we observed the BB0794 mutant to be more sensitive to lipid bilayer-permeating antibiotics such as tetracycline, minocycline and azithromycin, which could result from altered membrane permeability or biogenesis of the OM. It is also possible that the selective antibiotic sensitivities observed are the direct result of decreased efflux of the hydrophobic antibiotics after they enter the cell. It is known that bacterial efflux pumps, such as the one associated with TolC in *E. coli*, are responsible for resistance to multiple groups of antibiotics (Fralick, 1996; Zgurskaya and Nikaido, 2000; Sulavik *et al.*, 2001; Nikaido and Takatsuka, 2009). Along these lines, it is interesting that the TolC ortholog in *B. burgdorferi*, designated BesC, also has been shown to regulate sensitivity to many antimicrobials (Bunikis *et al.*, 2008). BesC forms the OM channel of the multi-drug efflux system in *B. burgdorferi* (Bunikis *et al.*, 2008) and BesC deletion mutants have been noted to be highly sensitive to hydrophobic antibiotics such as tetracycline and azithromycin (Bunikis *et al.*, 2008), which is consistent with what we observed in the BB0794 mutant strain. We have previously shown that the *B. burgdorferi* BAM complex is required for appropriate transport and OM integration of BesC into the borrelial OM (Lenhart and Akins, 2010; Dunn *et al.*, 2015). The observation that BB0794 and BamA interact, raises the intriguing possibility that BesC export or function is also altered in some fashion when BB0794/BamA interaction is diminished, which may result in decreased antibiotic efflux. Determining whether BB0794 is needed for appropriate export of a subset of OMPs or if BB0794 plays a role in regulating trafficking of lipid or protein components of the OM will be important questions to examine in future studies.

Although the TamA protein is found exclusively in *Proteobacteria*, TamB and TamB-like proteins are much more ubiquitous and are found in almost all diderms (Heinz *et al.*, 2015). TamA is typically encoded upstream of TamB in an operon (Gallant *et al.*, 2008; Selkrig *et al.*, 2012; Milner *et al.*, 2014; Heinz *et al.*, 2015); however, TamB-like proteins that neighbor BamA also have been identified in other diderms, such as the spirochete *B. burgdorferi* (Heinz *et al.*, 2015). The interaction between BB0794 and BamA is a novel observation suggesting that the TamA-TamB interaction in *Proteobacteria* is not the only significant role played by TamB-like proteins in diderms (Selkrig *et al.*, 2012; 2015; Shen *et al.*, 2014). The observation that BB0794 and BamA interact also could provide an explanation as to why so many diderms lacking TamA or autotransporter-like substrate OMPs, such as *B. burgdorferi*, contain a TamB-like protein. Interestingly, prior studies have also indicated that the substrate autotransporters of the TAM also require the BAM complex for complete autotransporter biogenesis (Jain and Goldberg, 2007; Sauri *et al.*, 2009; Rossiter *et al.*, 2011). Furthermore, it has recently been shown that the usher protein FimD from *E. coli* requires both the BAM complex and the TAM for efficient assembly of this protein into the outer membrane (Stubenrauch *et al.*, 2016). This previously reported dependence of substrate OMPs on both TAM and BAM also support our current model of a BB0794-BamA complex in ancestral spirochetal species that lack TamA. This also raises the important question as to whether TamB and TamB-like proteins from an organism that contains a TamA might also interact with BamA. Future studies to better define the interaction of TamB and TamB-like proteins with the BAM complex in diverse organisms should shed important light on the overall role of TamB in OM biogenesis beyond its interaction with TamA and OMP secretion in only a select group of *Proteobacterial* organisms.

Given that the DUF490 domain of TamB has been shown to interact with the POTRA domain of TamA (Selkrig *et al.*, 2012; 2015), we would propose that BB0794 in *B. burgdorferi* interacts with BamA through one or more of its POTRA domains. Lithgow and co-workers have reported that the β-barrel like strands of the DUF490 domain in TamB impart the interaction observed between TamB and the TamA POTRA domain (Heinz *et al.*, 2015; Selkrig *et al.*, 2015). They also proposed that this TamB interaction with TamA could help to regulate the activity of the TAM (Heinz *et al.*, 2015). Along similar lines of reasoning, it is tempting to speculate that BB0794 also maintains similar β-barrel like strand conformation in its DUF490 domain and this is responsible for the interaction between the BamA POTRA domain(s) and BB0794 in *B. burgdorferi*. An enhanced acknowledgment of the interaction between BB0794 and BamA and how it regulates the activities of the BAM complex will not only help us to better understand the concepts of spirochete physiology but it also could help to explain the paradoxical observation that while almost all diderms contain a TamB-like protein only a subset of organisms contain TamA.

Experimental procedures

Bacterial strains and growth conditions

All borrelial strains utilized in this study were cultivated at 34°C in BSK-II liquid medium containing 6% heat inactivated rabbit serum (complete BSK-II) with the addition of appropriate antibiotic(s) or isopropyl-β-D-thiogalactopyranoside (IPTG) as indicated. *B.*

burgdorferi B31 strain was used to perform cellular localization assays and co-immunoprecipitation experiments. *B. afzelii* Pko and *B. garinii* PBi (recently named B. bavariensis sp. nov. by Margos *et al.*, 2013) strains were for BB0794 expression immunoblot. The non-infectious *B. burgdorferi* B313 strain was used for expression of the $OspA^{WT}$ and $OspA^{794N}$ proteins from the shuttle vector pBSV2G (Elias *et al.*, 2003), which confers gentamicin resistance (40 μg ml^{-1}). The *B. burgdorferi* B31-5A4 LK strain expresses the LacI repressor and kanamycin resistance cassette from the endogenous plasmid lp25 (Gilbert *et al.*, 2007), and was used to generate the *flacp::bb0794* mutant. All shuttle vectors and constructs used for cloning were propagated using *E. coli* strain DH5α grown in lysogeny broth (LB) or on agar plates supplemented with the appropriate antibiotic(s).

Protein secondary structure and nucleotide sequence analyses of BB0794

The BB0794 signal peptide was predicted using PrediSi server (http://www.predisi.de/) (Nielsen *et al.*, 1997). Transmembrane α-helices in the BB0794 protein sequence were predicted with Phobius (http://phobius.sbc.su.se/) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM//) (Krogh *et al.*, 2001; Kall *et al.*, 2004). The secondary structure of BB0794 and *E. coli* TamB were predicted using PSIPRED v3.3 server (http://bioinf.cs.ucl.ac.uk/psipred/) (Jones, 1999). The promoter regions for *bb0794* and *bamA* were identified using BPROM (http://linux1.softberry.com/berry.phtml) and virtual footprint software v.3.0 (http://prodoric.tu-bs.de/vfp/vfp_promoter.php) (Munch *et al.*, 2005; V. Solovyev, 2011). Nucleotide sequence analysis of *bb0794* was determined from *B. burgdorferi* B31 genomic DNA using primers BB0794-2221F and BB0794-3297R (Table 2).

Generation of antibodies, immunoblot analyses, and triton X-114 phase partitioning

Antibodies specific to *B. burgdorferi* BB0794 were generated in rabbits against a synthetic peptide DTIGKEVQGLQ-LEIKGDDR (amino acids 345–363) by Thermo Fisher Scientific antibody production services (Thermo Scientific, Rockford, IL). Polyclonal antibodies against OspA, FlaB, P66, BamA, BamB, Skp, OppAIV or GST were generated in rats or rabbits as described previously (Radolf *et al.*, 1994; Brooks *et al.*, 2005; Brooks *et al.*, 2006; Lenhart and Akins, 2010; Kenedy *et al.*, 2014). A monoclonal antibody specific to LacI was used according to manufacturer's instructions (US Biological, Swampscott, MA).

For all immunoblots, samples were subjected to separation by 10% SDS-PAGE before being transferred to polyvinylidene difluoride (PVDF) membranes. Membranes with transferred proteins were subsequently blocked for 45 min in milk buffer comprised of phosphate buffered saline (PBS), 5% nonfat dried milk, and 0.5% Tween. After incubation with the primary antibody for 1–2 h, membranes were washed three times with milk buffer and subsequently incubated with HRP-conjugated goat anti-rat or goat anti-rabbit antibodies (Invitrogen, Carlsbad, CA) at 1:10 000 dilution for one hour. Following incubation, membranes were washed three times with milk buffer and twice with PBS before being developed using SuperSignal West Pico enhanced chemiluminescent reagent according to the manufacturer's instructions (Thermo Scientific). The images were captured using a Fluor Chem Q instrument with associated AlphaView analysis software (Alpha Innotech, San Leandro, CA)

Triton X-114 phase partitioning was performed on whole cells from *B. burgdorferi* strain B31 or strain B313 expressing the OspA^{794N} fusion or on the purified recombinant OspA protein lacking its signal peptide (Weigel *et al.*, 1992; Radolf *et al.*, 1994). All phase partitioning experiments were performed as previously described (Bordier, 1981; Brusca and Radolf, 1994; Brooks *et al.*, 2006).

BB0794 surface localization assays

Indirect immunofluorescence assays (IFA) were performed as described previously (Dunn *et al.*, 2015). Briefly, *B. burgdorferi* B31 cultures were diluted to 5×10^6 organisms ml^{-1} in complete BSK-II medium before cells were either incubated in solution with antibodies or spotted onto glass slides and fixed with acetone. Organisms in solution or fixed on slides were then incubated with a mixture of either rat anti-FlaB (1:500) with rabbit anti-BB0794 (1:100) antibodies or a mixture of rabbit anti-FlaB (1:500) with rat anti-OspA (1:500) antibodies. Organisms in solution were then washed, spotted on slides, and allowed to dry before secondary antibodies were added. For the BB0794 experiments a combination of Alexa-Fluor 488-conjugated goat anti-rabbit and Alexa-Fluor 568-conjugated goat anti-rat antibodies secondary antibody (Life Technologies, Grand Island, NY) was added at a 1:500 dilution and for the OspA experiments a combination of Alexa-Fluor 488-conjugated goat anti-rat and Alexa-Fluor 568-conjugated goat anti-rabbit antibodies was used at a 1:500 dilution. The spirochetes were visualized at 1000× magnification using an Olympus BX60 fluorescent microscope (Olympus America, Inc., Center Valley, PA).

Proteinase K (PK) surface accessibility assays were performed on B31 strain and B313 strain expressing OspA^{794N} and OspA^{WT} as described previously (Brooks *et al.*, 2006).

Membrane fractionation

Highly enriched outer membrane (OM) and protoplasmic cylinder (PC) fractions of *B. burgdorferi* strain B31 were obtained using sucrose density gradient separation as described (Skare *et al.*, 1995; Mulay *et al.*, 2007; Lenhart and Akins, 2010; Dunn *et al.*, 2015). All fractionations were performed on a starting amount of 5×10^{10} mid-log phase organisms (1 L of 5×10^7 ml^{-1}).

Generation of the BB0794/OspA fusion protein (OspA^{794N})

To examine the physicochemical properties of the predicted N-terminal signal peptide of BB0794, a chimeric protein encoding the first 39 amino acids of BB0794 was fused to the mature portion of OspA lacking its signal peptide. The resulting chimera, designated OspA^{794N}, included nucleotides 1–117 of *bb0794*, which was amplified using *B. burgdorferi* genomic DNA and primers BB0794-1F and BB0794-117R (Table 2). The resulting amplicon was digested using restriction enzymes BamHI and XbaI and ligated into the borrelial shuttle vector pBSV2G (Elias *et al.*, 2003). Next, nucleotides 49–822 of the *ospA* gene were PCR amplified using primers OspA-49F and OspA-822R (Table 2) and the amplicon was digested with restriction enzymes XbaI and SalI before being ligated downstream of *bb0794* insert in pBSV2G. The *flgB* promotor was then amplified using primers FlgB-1F and FlgB-407R (Table 2), digested with KpnI and BamHI before being cloned immediately upstream of the *bb0794/ospA* chimera. The resulting construct

expresses the N-terminal 39 amino acids from BB0794 fused to the mature portion of OspA corresponding to amino acids 17–274. The construct $OspA^{WT}$ was generated by amplifying the entire *ospA* gene (nucleotides 1–822) using primers OspA-1F and OspA-822R (Table 2). The amplicon was digested and ligated into pBSV2G downstream of the *flgB* promotor that was inserted into pBSV2G as outlined above. The final $OspA^{794N}$ and $OspA^{WT}$ constructs were verified by sequence analysis to contain no mutations and electroporated into *B. burgdorferi* strain B313 to further examine the properties of the BB0794 N-terminus.

Co-immunoprecipitation (co-IP) assays

Co-IP experiments were performed using the Pierce Direct IP Kit (Thermo Scientific, Rockford, IL). Briefly, *B. burgdorferi* B31 cells were grown to mid-log phase (5×10^7 organisms ml^{-1}) in complete BSK-II medium before whole-cell lysates were prepared as previously described (Lenhart *et al.*, 2012). 500 μl of final lysates were then mixed with AminoLink resin with conjugated BB0794, BamA, or GST antibodies following the manufacturer's instructions (Thermo Scientific). The lysate and resin mixture was rotated end over end at 4°C overnight before being washed. Proteins bound to the resin were eluted by adding 50 μl of final sample buffer containing β-mercaptoethanol and boiled for seven minutes before SDS-PAGE and immunoblot analysis.

Generation of a BB0794 IPTG regulatable mutant

The BB0794 regulatable mutant, designated *flacp::bb0794*, was generated in the *B. burgdorferi* B31-5A4 LK strain generously provided by Dr. D. Scott Samuels (University of Montana) (Gilbert *et al.*, 2007). *B. burgdorferi* genomic DNA was first used as template to amplify 600 bp regions upstream and downstream of the start codon of the *bb0794* gene using primers *flacp::bb0794*-831046F and *flacp::bb0794*-831645R (upstream region) and *flacp::bb0794*-4F and *flacp::bb0794*–600R (downstream region) as indicated in Table 2. The two 600 bp amplicons were directionally cloned into the pBluescript-II KS+ vector (Stratagene, La Jolla, CA) using the restriction sites added into the amplification primers. Subsequently, the streptomycin resistance cassette and hybrid *flacp* promotor were digested as a unit from the previously described *flacp*::BB0028 plasmid construct (Dunn *et al.*, 2015) using restriction enzymes XhoI and NdeI before it was ligated between the two 600 bp amplicons that flank the BB0794 start site. The resulting recombination construct was then electroporated into the B31-5A4 LK strain and incubated in complete BSK-II supplemented with kanamycin (200 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and IPTG (1 mM). Two weeks later, electroporated cultures in 96-well plates were screened for presence of antibiotic-resistant transformants. One clone identified, designated *flacp::bb0794*, was confirmed by PCR analysis to have undergone homologous recombination using the primers indicated in Table 2. The mutant was subsequently subjected to a complete plasmid analysis as previously described (Elias *et al.*, 2002; Lenhart and Akins, 2010) before IPTG dose-dependent regulation of BB0794 was examined and confirmed by immunoblot analysis (see Fig. 6C).

Transcriptome analysis

Total RNA was isolated from *B. burgdorferi* strain B31-5A4 using a hot phenol protocol as described (Lybecker *et al.*, 2014). The isolated RNA was DNase I treated (Roche,

Indianapolis, IN) and depleted of ribosomal RNA using the Ribo-Zero RNA removal kit for Gram-negative bacteria following the manufacturer's protocol (Epicentre, Madison, WI). RNA integrity was measured using an Agilent 2100 Bioanalyzer and RNA with an RNA Integrity Number (RIN) above 9.0 was used for subsequent cDNA library construction. Directional (strand-specific) RNA-seq cDNA libraries were constructed with a ligation-based protocol as previously described, but with an initial size-selection (Lybecker *et al.*, 2014). Briefly, the fragmented RNAs were treated to remove 5′ and 3′ phosphates and 2′-3′ cyclic-phosphates and subsequently 3′ and 5′ RNA adapters were ligated at both ends as described (Lybecker *et al.*, 2014). A size selection of total RNA correlating to 50–500 nucleotides was conducted before fractionation on an 8% TBE-UREA gel. The di-tagged RNA libraries were then gel-purified and reverse transcribed for PCR analysis and Solexa sequencing as described (Lybecker *et al.*, 2014). Sequences have been deposited at the NCBI Sequence Read Archive under study accession number SRP078488 and experiment accession numbers SRX1948239 and SRX1948242.

Growth curve analysis

Growth curves were performed in triplicate as previously described (Lenhart and Akins, 2010). The parental B31-5A4 LK strain was passed into 14 ml tubes of prewarmed BSK-II complete media containing kanamycin (200 μg ml^{-1}) and seeded at 3000 organisms ml^{-1}. The *flacp::bb0794* mutant was first washed with complete BSK-II to remove residual IPTG before seeding 3,000 organisms ml^{-1} in BSK-II complete media containing kanamycin (200 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and a final IPTG concentration of 0 mM, 0.05 mM or 1.0 mM. Spirochetes were enumerated using dark-field microscope every 24 h for 11 days. Statistical significance was determined using unpaired t test (two-tailed) with Welch's correction.

Antimicrobial susceptibility assays

Antimicrobial susceptibility of the parental strain B31-5A4 LK (WT) was compared to the *flacp::bb0794* mutant cultivated with or without IPTG as previously described (Bunikis *et al.*, 2008; Dunn *et al.*, 2015) using the following antibiotics and ranges (ng ml^{-1}): penicillin (10–20,000), carbenicillin (2–5,000), cefotaxime (25–50,000), tetracycline (5–10,000), minocycline (5–10,000), and azithromycin (1–2,000). *B. burgdorferi* growth was assessed in differing concentrations of all antibiotics at 0, 24, 48 and 72 h by measuring the absorbance of each well at 562 and 630 nm using a Molecular Devices SpectraMax 340 plate reader (Molecular Devices, Sunnyvale, CA). Each assay was repeated four times in duplicate and the mean 562/630 nm ratio, which corresponds with the amount of borrelial growth per well, for all concentrations of each antibiotic was plotted with respect to time to determine the minimum inhibitory concentration (MIC) of each antibiotic.

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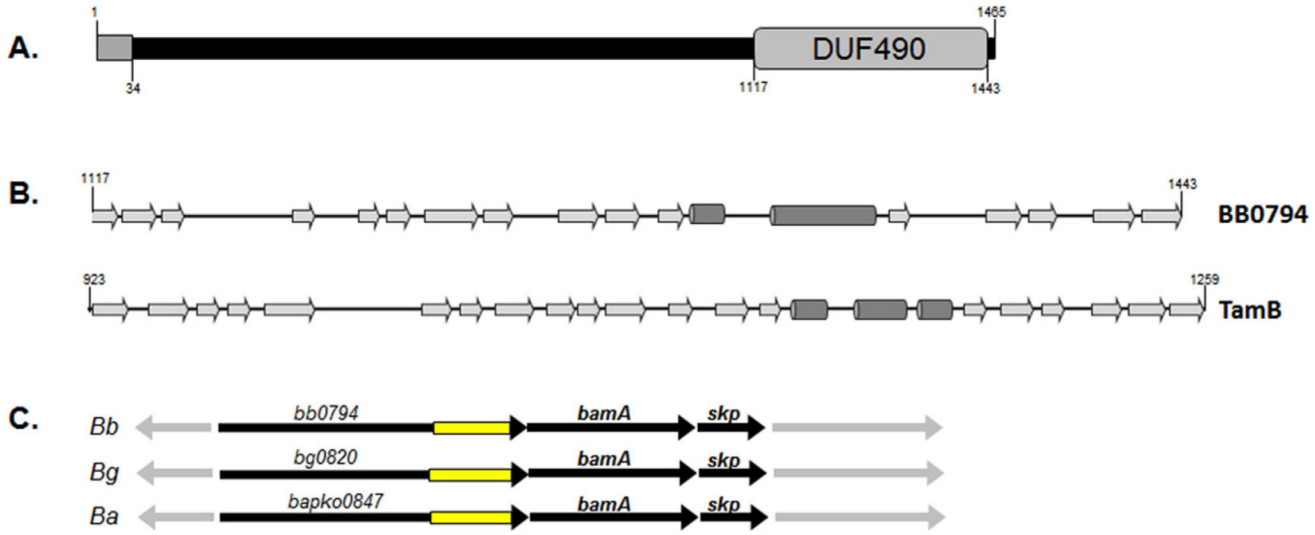

Fig. 1. Domain analysis and secondary structure prediction of BB0794 and its genetic organization in *B. burgdorferi* sensu lato

A. Schematic of the BB0794 protein. The gray boxes from amino acids 1–34 indicate the putative N-terminal signal peptide predicted by PrediSi (Nielsen *et al.*, 1997) while amino acid 1117–1443 represent the C-terminal DUF490 domain identified by the Conserved Domain Database (CDD) algorithm (Derbyshire *et al.*, 2015).

B. Comparison of the *B. burgdorferi* BB0794 DUF490 domain (amino acids 1117–1443) and the *E. coli* TamB DUF490 domain (amino acids 923–1259). Secondary structure was predicted using PSIPRED (Jones, 1999) with β-sheet and α-helical regions equal to or greater than five amino acids designated as arrows and cylinders respectively.

C. Genetic organization of the chromosomal region surrounding the *B. burgdorferi* (*Bb*) *bb0794* gene and the corresponding genomic region in *B. garinii* (*Bg*) and *B. afzelii* (*Ba*). Yellow boxes indicate the predicted DUF490 domains.

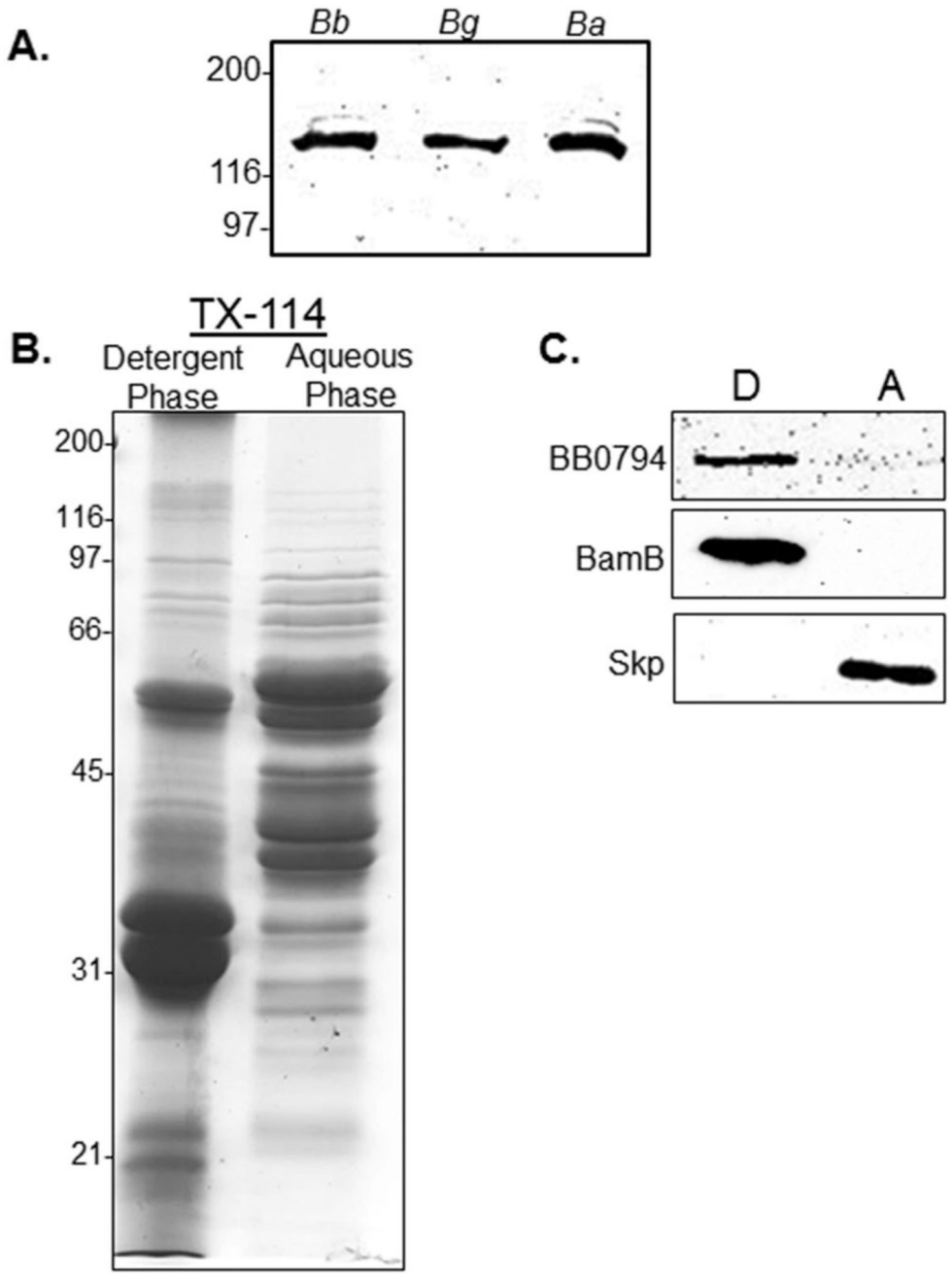

Fig. 2. Expression and physicochemical properties of BB0794

A. Immunoblot analysis of whole-cell lysates (5×10^7 per lane) of *B. burgdorferi* (*Bb*), *B. garinii* (*Bg*) and *B. afzelii* (*Ba*) using BB0794-specific antisera. Molecular weight markers, in kDa, are shown at left.

B. Triton X-114 phase partitioning (TX-114) of *B. burgdorferi* with equivalent amounts of the detergent-enriched and aqueous-enriched phases separated by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) R-250. Molecular weight standards, in kDa, are shown at left.

C. Equal amounts of the detergent-enriched (D) and aqueous-enriched (A) phases were subjected to immunoblot analysis with BB0794-specific antisera. To ensure phase separation was complete, immunoblots with specific antisera against the amphiphilic membrane lipoprotein BamB and the soluble periplasmic protein Skp were included as controls.

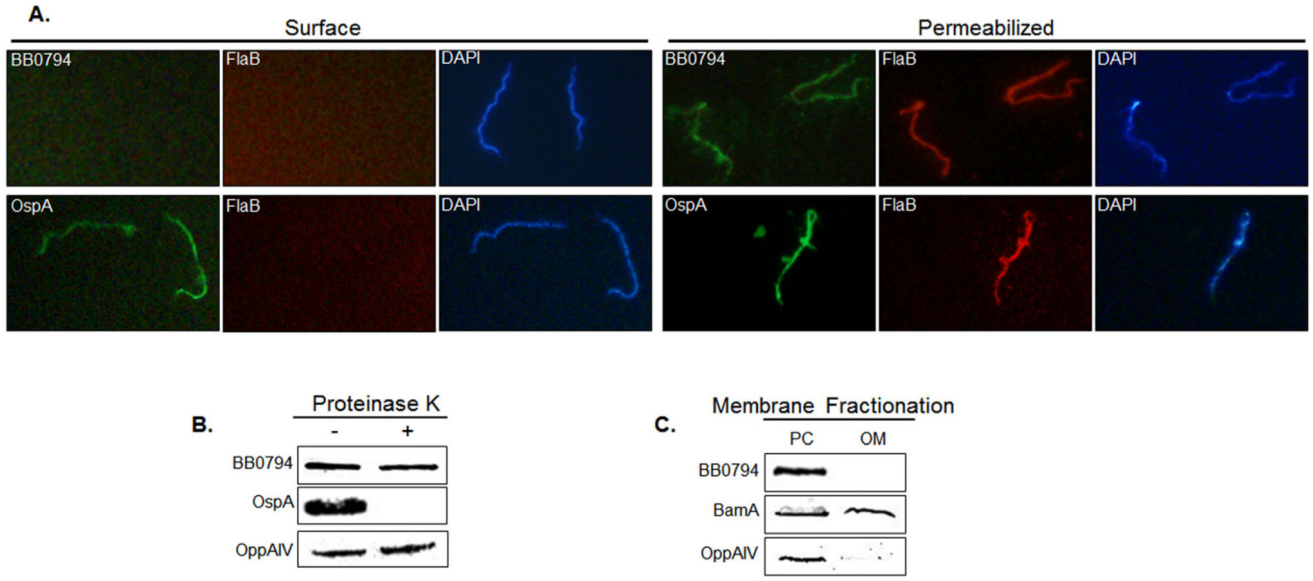

Fig. 3. BB0794 is not surface-exposed and associates with the IM

A. Indirect immunofluorescence assays were performed in solution on intact *B. burgdorferi* (Surface panels) and acetone-fixed organisms (Permeabilized panels). Spirochetes were probed with BB0794-specific antisera (BB0794 panels) or antibodies directed against the surface exposed protein OspA (OspA panels) along with antibodies against the periplasmic protein FlaB (FlaB panels). FlaB antibodies were included with the BB0794 or OspA antibodies to ensure that organisms remained intact during the surface immunofluorescence assays. DAPI (4′,6-diamidino-2-phenylindole) was also included for each experiment as a counterstain to identify all spirochetes in a given field.

B. *B. burgdorferi* whole-cell lysates (1.25×10^7 per lane) were incubated with (+) or without (−) proteinase K before being subjected to immunoblot analysis with specific antibodies against BB0794, OspA, or OppAIV.

C. Protoplasmic cylinder (PC) or outer membrane (OM) fractions from *B. burgdorferi* were isolated before equivalent amounts were subjected to immunoblot analysis with antibodies specific for BB0794, BamA, or OppAIV.

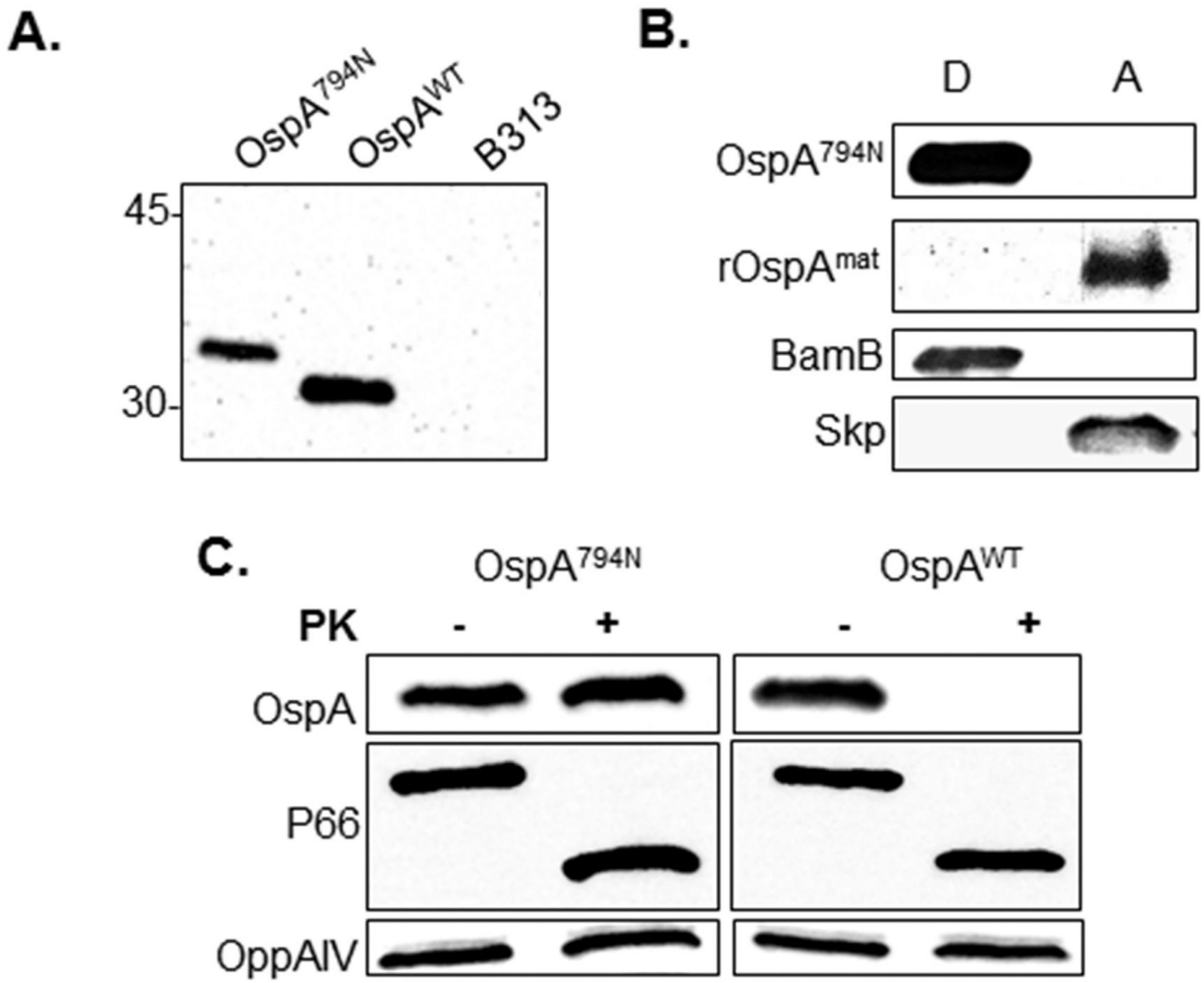

Fig. 4. BB0794 encodes an uncleaved signal peptide membrane anchor

A. Whole-cell lysates of *B. burgdorferi* strain B313 expressing either the N-terminal 39 amino acids of BB0794 fused to OspA lacking its signal peptide (OspA^{794N}) or the wildtype OspA (OspA^{WT}) protein were subjected to immunoblot analysis using OspA specific antisera. Whole cell-lysates of B313, which lacks the lp54 plasmid encoding OspA, was used as a negative control. Molecular weight standards, in kDa, are indicated at left.

B. Triton X-114 phase partitioning was performed on whole-cell lysates of B313 strains expressing the N-terminal 39 amino acids of BB0794 fused to the OspA protein lacking its signal peptide (OspA^{794N}) and a recombinant form of OspA lacking its signal peptide (rOspA^{mat}). The detergent-enriched (D) and aqueous-enriched (A) phases were separated by SDS-PAGE and subjected to immunoblot using OspA-specific antibodies. Additionally, antibodies specific to the membrane-anchored BamB lipoprotein and periplasmic protein Skp were included as controls to ensure complete phase separation of the borrelial whole-cell lysates.

C. B. burgdorferi strain B313 expressing OspA^{794N} or OspA^{WT} were incubated with (+) or without (−) proteinase K (PK) before whole-cell lysates (1.25×10^7 per lane) were subjected

to immunoblot analysis with specific antibodies against OspA, the known outer membrane protein P66, or the inner membrane lipoprotein OppAIV.

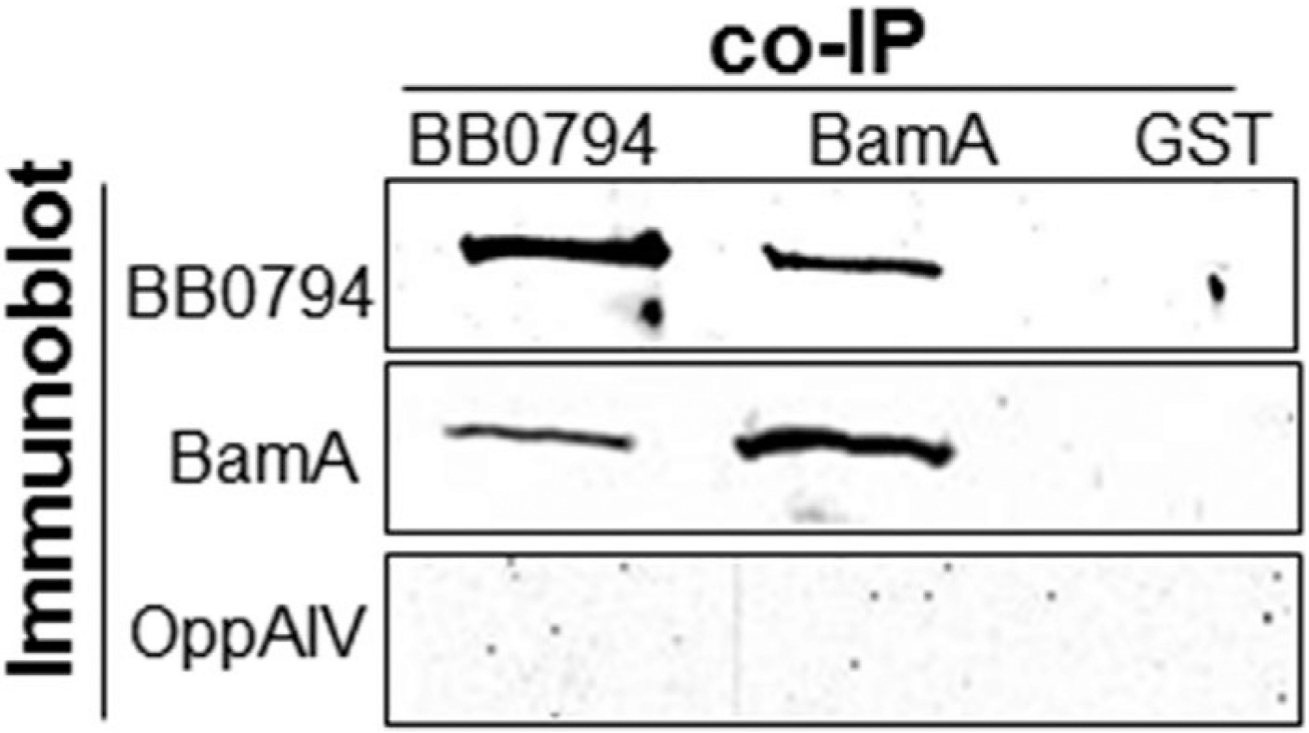

Fig. 5. BB0794 and BamA can be co-immunoprecipitated in *B. burgdorferi*

Cultures of *B. burgdorferi* B31 (1×10^{10} organisms) were subjected to co-immunoprecipitation (co-IP) experiments with BB0794 and BamA-specific antibodies (indicated above top panel). Equal amounts of each co-IP elution were then subjected to SDS-PAGE and immunoblot analysis using antisera generated against BB0794 and BamA (indicated at left of each panel). To illustrate specificity of the BB0794-BamA interaction, elutions were also immunoblotted with antibodies against the inner membrane lipoprotein, OppAIV (bottom panel). GST-specific antibodies were included in the co-IP experiments as a nonspecific antibody control (right lane of each panel).

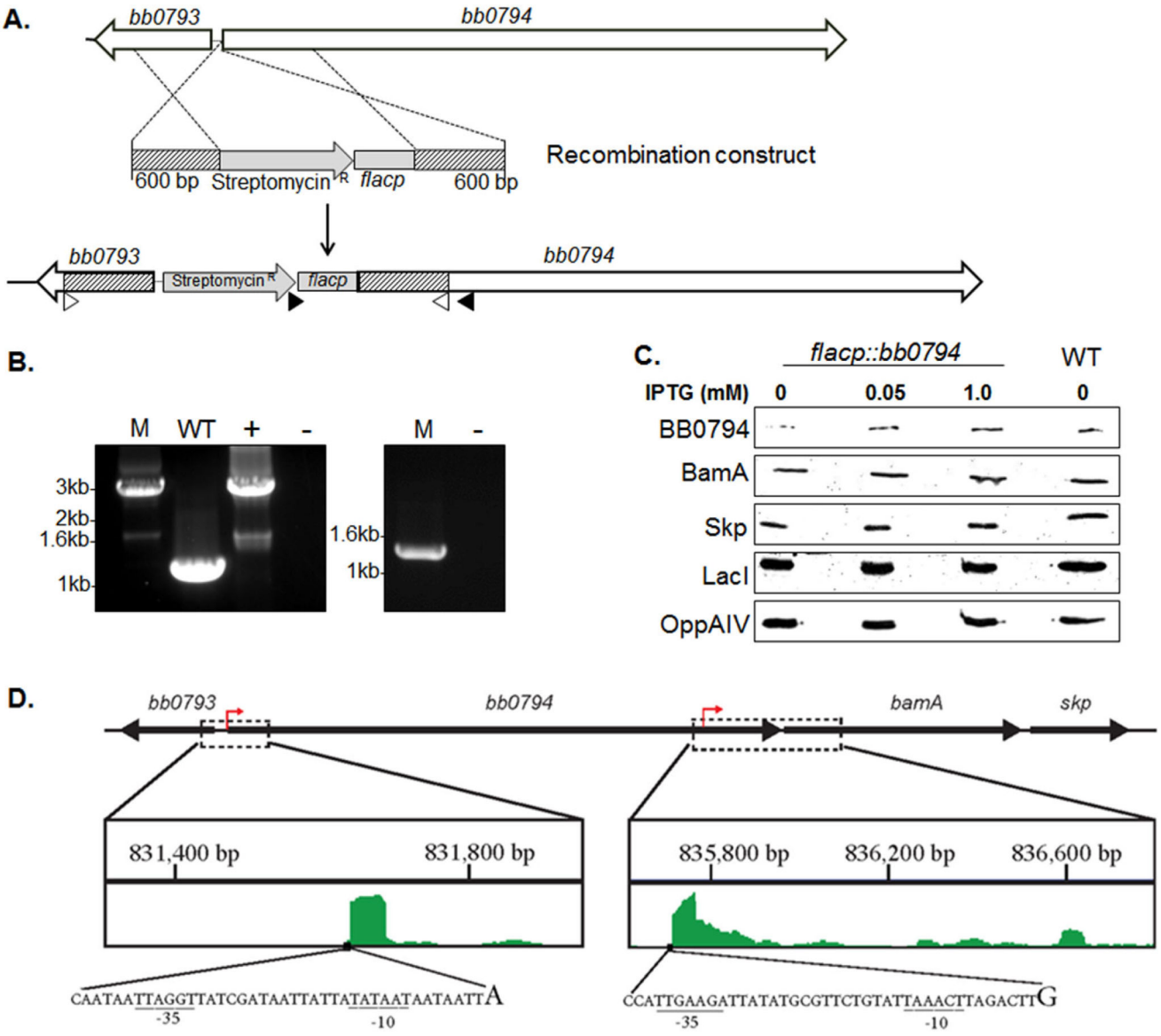

Fig. 6. Construction of an IPTG-regulated *bb0794* gene and identification of unique transcriptional start sites for *bb0794* and *bamA/skp*

A. The promoter region upstream of the *bb0794* gene was replaced with the *flacp* IPTG-regulatable promotor and a streptomycin resistant cassette in *B. burgdorferi* strain B31-5A4 LK by homologous recombination as outlined in the Experimental Procedures.

B. PCR analysis of DNA from the resulting mutant *flacp::bb0794* (M) and the parental strain B31-5A4 LK (WT) verified insertion of the complete recombination construct (left panel) and the orientation of the construct in the resulting mutant (right panel). Shown in the left panel are the amplicons resulting from the primer pair indicated by white arrowheads in Panel A that encompass the complete region inserted by the recombination construct. As expected, the 3 kb amplicon from the *flacp::bb0794* mutant (lane M), which includes the streptomycin cassette and *flacp*, was 1.8 kb larger than the amplicon from the parental strain, which resulted in a 1.2 kb amplicon (lane WT). The recombination construct cloned into

pBluescript-II KS+ was used as a positive control (+) while a reaction with no template DNA added was used as a negative control (−). Shown in the right panel is the amplicon generated from the primer pair shown as black arrowheads in Panel A that includes a region outside of the recombination construct to confirm the orientation of the streptomycin cassette and *flacp* promoter (lane M). A negative control was included lacking template DNA (−). Molecular weight standards for both panels, in kb, are shown at left.

C. Whole-cell lysates (5×10^7) of the *flacp::bb0794* mutant cultivated in 0, 0.05, and 1 mM IPTG or the parental strain B31-5A4 LK (WT) were subjected to immunoblot analysis using BB0794, BamA, Skp, LacI, or OppAIV specific antibodies.

D. Identification of the mRNA start sites in the region flanking *bb0794*. Transcriptome data were mapped to the *B. burgdorferi* genome and visualized via Integrative Genomics Viewer (IGV). The genomic context of the operon is illustrated with transcriptional start sites (TSS) indicated by red arrows. The dashed box indicates the region of the gene displayed in the expanded coverage map of the transcriptome data generated. Putative promoter sequences (−10 and −35 sites) upstream of *bb0794* and *bamA* are shown below the coverage maps and were predicted using BPROM (V. Solovyev, 2011) and virtual footprint v.3.0 (Munch *et al.*, 2005). The specific transcriptional start sites are represented by nucleotides in large, bold font for *bb0794* (**A**) and *bamA* (**G**).

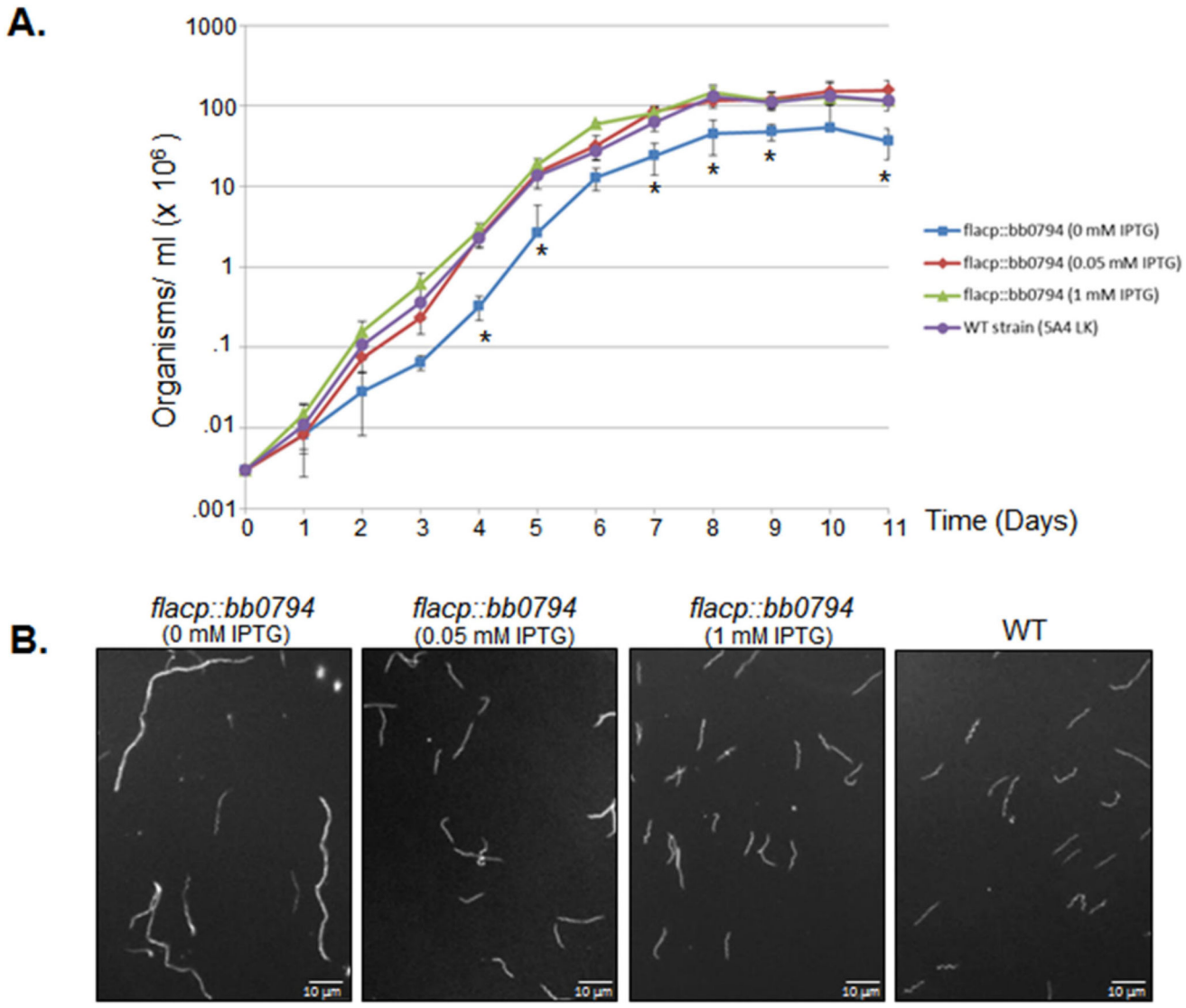

Fig. 7. Growth and phenotypic analysis of the flacp::bb0794 mutant

A. The *flacp::bb0794* mutant was grown in BSK-II complete medium in the absence of IPTG or in the presence of 0.05 mM or 1 mM IPTG along with the parental, strain B31-5A4 LK (WT). Triplicate samples for each growth condition were seeded at 3 000 organisms ml^{-1}. Spirochetes were enumerated by dark field microscopy every 24 h for 11 days and mean number of organisms are shown. Significant differences between *flacp::bb0794* grown in the absence of IPTG and all other culture conditions was assessed using t-test with Welch correction (*p* values < 0.05 were considered significant) and are indicated by asterisk. Error bars represent standard deviation.

B. The *flacp::bb0794* mutant cultivated in complete BSK-II without IPTG or with 0.05, and 1 mM IPTG and the parental strain B31-5A4 LK (WT) were examined by dark-field microscopy at 400× magnification. Shown are representative fields of spirochetes from each condition during the mid-logarithmic phase of growth.

Table 1

BB0794 depletion alters the *B. burgdorferi* antibiotic sensitivity profile.

	Minimum inhibitory concentration (MIC) ng ml^{-1}					
Strain	**Tetracycline**	**Minocycline**	**Azithromycin**	**Penicillin G**	**Carbenicillin**	**Cefotaxime**
flacp::bb0794 (0 mM IPTG)	625 (**2×**)	20 (**2×**)	4 (**2×**)	625	625	98
flacp::bb0794 (1 mM IPTG)	1250	40	8	625	625	98
WT	1250	40	8	625	625	98

Table 2

Oligonucleotides used in this study.

Primer name	Primer sequence (5′ to 3′)[a]	Primer details
BB0794-2221F	TTAGGGGTTAATTCCGATGAATATGG	BB0794 nucleotides 2221–2246; Sequence verification of BB0794
BB0794-3297R	GCG**CTCGAG**TCCCGTTCTACCAGTCACAT	Complementary to BB0794 nucleotides 3278–3297 plus XhoI restriction site; Sequence verification of BB0794
flacp::bb0794-831046F	GCG**GGTACC**TCTAGTTTTTTAAATATTTTTAAATATTTT	Corresponding to nucleotides 831,046-831,075 of *B. burgdorferi* chromosomal genome plus KpnI restriction site
flacp::bb0794-831645R	CGC**CTCGAG**ACACTCTATCGATAATTATTATTATAT	Corresponding to nucleotides 831, 619–831,645 of *B. burgdorferi* chromosomal genome plus XhoI restriction site
flacp::bb0794-4F	GCG**GTCGACCATATG**AATTTGTTGTTTTTGAGAAGTAAG	BB0794 nucleotides 4–27 plus SalI and NdeI restriction sites
flacp::bb0794–600R	CGC**GGATCC**ATTTTTAACAGCACTAAAATCAAC	Complementary to BB0794 nucleotides 577–600 plus BamHI restriction site
aadA1-766F	GCGGAGATCACCAAGGTAGTCGGCAAA	Nucleotides 766–789 of *aadA1* gene; PCR verification of *flacp::bb0794*
BB0794-927R	ATAAATTTCAAAGCCTTTGTTTAGCTTAATCCAATC	Complementary to BB0794 nucleotides 892–927; PCR verification of *flacp::bb0794*
FlgB-1F	GCG**GGTACC**TACCCGAGCTTCAAGGAAGAT	flgB nucleotides 1–21 plus KpnI restriction site
FlgB-407R	CGC**GGATCC**ATGGAAACCTCCCTCATTTAAAATT	Complementary to flgB nucleotides 383–407 plus BamHI restriction site
BB0794-1F	GCG**GGATCC**ATGAATTTGTTGTTTTTGAGAAGTAAG	BB0794 nucleotides 1–27 plus BamHI restriction site
BB0794-117R	CGC**TCTAGA**TGCAGAATAAATTTGAGCTTGAAC	Complementary to BB0794 nucleotides 94–117 plus XbaI restriction site
OspA-1F	GCG**GGATCC**ATGAAAAAATATTTATTGGGAATAGGT	OspA nucleotides 1–27 plus BamHI restriction site
OspA-822R	CGC**GTCGAC**TTATTTTAAAGCGTTTTTAATTTCATCAAG	Complementary to OspA nucleotides 793–822 plus SalI restriction site
OspA-49F	GCG**TCTAGA**TGTAAGCAAAATGTTAGCAGCCTT	OspA nucleotides 49–72 plus XbaI restriction site

[a] Restriction sites shown in bold.