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RESEARCH ARTICLE

Conserved hypothetical BB0462 protein enhances the transcription activity of *opp*AV promoter

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Abstract *Borrelia burgdorferi* BB0462 ORF encodes an unknown functional protein with 110 amino acids. A BLAST search in protein databases and the secondary structure being predicted by the program JUFO showed that the conserved hypothetical BB0462 protein was similar to the members of the YbaB protein family in both amino acid composition and protein structure. The co-transformation of BB0462 ORF and *opp*A upstream regulation DNA into *E. coli* host cells and β-galactosidase activity assay demonstrated that the BB0462 protein enhanced the transcriptional activity of the *opp*AV promoter, but does not affect those of *opp*AI, II, III and IV promoters. Analysis of DNA retardation and competitive repression also confirmed that the BB0462 protein bound to the 409 bp upstream regulation DNA fragment close to the initiation codon of the *opp*AV gene. All data in our study suggested that the BB0462 protein was involved in the transcriptional regulation of the *opp*AV gene

Keywords BB0462 protein, *opp*AV promoter, gel mobility shift assay, transcriptional activator

1 Introduction

Borrelia burgdorferi is the causative agent of human Lyme disease (Johnson et al., 1984). Its genome is unusual in genes devoted to the biosynthesis of amino acids, nucleic acids and fatty acids. *Borrelia burgdorferi* survives by acquiring external nutrients, for example the N source acquired by oligopeptide permease (Opp). Opp belongs to the ABC-type transporters with a peptide-binding protein interacting with two inner membrane transmembrane proteins and two ATP-binding proteins. The *B. burgdorferi* genome encodes five separate oligopeptide-binding proteins (OppA), OppA1, OppA2, OppA3, OppA4 and OppA5. Each of these appears to be capable of integrating membrane proteins of the transporter. Three of these genes ecoding OppA, *opp*AI, *opp*II and *opp*III, are clustered in the *opp* operon located in the chromosome, and two are on distinct plasmids (*opp*AIV on cp26 and *opp*AV on lp54) (Fraser et al., 1997). Previous functional tests demonstrate that all five OppAs are capable of facilitating the transport of small peptides (Lin et al., 2001) and have overlapping but distinct substrate preferences (Wang et al., 2004). The functional assays of the upstream DNA fragments of five *opp*A genes also reveal that each *opp*A gene has its own promoter regulating independently its downstream *opp*A gene although each promoter activities are different (Wang et al., 2002). It has been found that the *opp*AI promoter appears to be constitutive, while *opp*AII-V promoters are inducible. The transcriptional activities of *opp*AIV and *opp*AV in mice are significantly higher than those in tick or *in vitro* cultivation (Wang et al., 2002).

Many open reading frames (ORF) are able to encode the proteins with a small molecular weight and unknown biological function in the *B. burgdorferi* genome (Fraser et al., 1997). These small proteins play a role in regulating the gene expression in the life cycle of *B. burgdorferi*. For instance, BB0647 encodes a protein with Fur function, which may regulate the oxidative stress in the *B. burgdorferi* cells as a repressor (Boylan et al., 2003; Katona et al., 2004). Here, the relationship between BB0462 protein and promoters of five *opp*A genes were analyzed. It is found that BB0462 protein can enhance the transcriptional activity of these five *opp*A promoters.

2 Materials and methods

2.1 Plasmid transformation and BB0462 protein expression

The recombinant plasmid pET30a/BB0462 was transformed into *E. coli* BL21(DE3) by electroporation. The

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transformants were initially selected by using LB plates containing 50 mg/mL kanamycin and then further confirmed by PCR. To induce gene expression in the transformed cells, IPTG was added to the final concentration of 0.5 mM. After incubating at 37°C for 12 h, bacterial cells were harvested by centrifugation. Cell pellets were re-suspended in 50 mM phosphate buffer (pH7.4) containing 100 mM NaCl and 1 mM DTT. Bacterial cells were broken via sonication, and crude extracts were prepared and clarified by centrifugation at 2000 g for 15 min at 4°C.

2.2 Western blotting

The crude extract was separated by 10% SDS-PAGE, and the proteins on the gel were transferred to immobilon™-P membrane. The membrane was socked in TBST (25 mM Tris-HCl (pH7.6), 150 mM NaCl and 0.1% Tween-20) at room temperature for 1 h, and then blocked by adding 5% non-fat milk for 1 h. In view of the fact that the BB0462 protein carries the influenza virus hemagglutinin (HA) epitope tag at its N-terminal, the anti-HA antibody conjugated with HRP was used for hybridization with the membrane at room temperature for 1 hour. Then, the membrane was washed with TBST for three times. The result for hybridization was detected through X-ray exposure after the mixture of 1 × LumiGLO and peroxide interacted with the membrane for 5 min. For the detailed procedure, the reader is referred to the instruction of Cell Signaling Technology.

2.3 Protein purification

The crude extract was mixed with 5 mL Talon resin (BD Bioscience) and then shaken gently at 4°C overnight. The resin was collected by centrifugation and washed with potassium phosphate buffer (pH8.0) containing 200 mM NaCl for three times. Then, the resin was packed into a column and washed again with the same buffer. The 20 mM imidazole in the same buffer was used to remove non-specific binding proteins from the resin, and 200 mM imidazole was finally used to elute the target protein out of the column. Protein purity was routinely examined by using 10% SDS-PAGE. Protein concentration was determined using a Bio-Rad assay kit, in which BSA was used as standard protein.

2.4 Gel shift assay

The upstream regulation regions of *B. burgdorferi* genes *opp*AI–V were individually amplified according to the procedure described previously (Wang et al., 2002). The DNA fragments amplified by PCR were purified using a PCR DNA purification kit (Qiagen). Gel shift assay was performed according to the procedure described by Carina Holmberg on the Internet (www. biochem. northwestern.edu). The DNA fragments were mixed with 12.5 μL 2× binding buffer, 5 μL buffer C, 1 μL 10 g/L BSA, 1 μL 6 μM purified protein, and the total volume was controlled at 25 μL. After storing at room temperature for 30 min, the mixture was separated by 4% non-denatured PAGE. When ethidium bromide (EB) was used to detect DNA in the gel, 2 μg/μL DNA fragments were used. The result of the gel shift was observed directly under UV light and photographed. When DIG-labeled DNA fragments were used in the gel shift assay, 15 fmol/μL DNA was applied. Because the anti-DIG antibody was conjugated with alkaline phosphatase, the chemiluminescent substrate CSPD (applied Biosystems) was used in the assay. For the detailed procedure, the reader is referred to the instruction for gel shift assay kit (Roche).

2.5 *β*-Galactosidase activity assays

The upstream regions of *opp*AI–V were respectively inserted into the *Bam*HI and *Bgl*II sites of pCB182 plasmid as described previously (Wang et al., 2002). Five recombinant plasmids with the upstream regions of *opp*A gene were separately co-transformed into *E. coli* BL21 $(DE)_3$ with pET30a/BB0462 plasmid. The transformants were selected by growing bacterial cells on LB plates containing 100 mg/mL ampcillin and 50 mg/mL kanamycin. Positive colonies were further confirmed by PCR. *β*-galactosidase activity assay was performed according to the method described previously (Wang et al., 2002). The co-transformed *E. coli* $BL21(DE)_3$ cells containing pET30a and the recombinant plasmid pCB182 carrying the upstream region of either *opp*AI, II, III, IV or V was used as the control. *β*-galactosidase activity was monitored by measuring the absorbance at the wavelength of 420 nm and 550 nm. Enzyme activity was calculated based on the equation: units of activity $(U) = 1000 \times [OD_{420} - 91.75 \times OD_{550}] / (\text{time} \times \text{volume} \times OD_{600})$. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μM O-nitrophenyl-*β*-D-galactopyranoside per minute (Miller, 1972).

3 Results

3.1 BB0462 protein possessing the secondary structure similar to those of transcription activators

B. burgdorferi BB0462 ORF encodes a conserved hypothetical protein with a molecular mass of 12.48 kDa. This lysine-rich protein, with 16 lysine residues out of 110 amino acids, has a putative pI value of 6.29, but its biological function is unknown. A BLAST search in protein databases found that the BB0462 protein is similar to the ones in the YbaB family with a similarity of 95%. The crystal structure of YbaB protein from *Haemophilus influenzae* was resolved although the protein function in the YbaB family was unknown. The molecular structure of *H. influenzae* YbaB protein was found to be similar to those of HLH type of transcription activators in eukaryotes. Each monomer is composed of two longer α-helices close to the two terminuses of the protein and two smaller β-sheets in the middle of the protein, and two monomers dimerize a tongue-like structure (Lim et al., 2003). The secondary structure of BB0462 protein was computed by JUFO (www.jens-meiler.de/jufo.html) based on its amino acid sequences, and the result is shown in Fig. 1. The BB0462 protein in Fig. 1 shows two smaller β-sheets flanked by two longer α−helices, which indicate that the BB0462 protein could form a tongue-like dimer structure and might act as a DNA-binding protein.

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---------------------HHHHHHHHHHHHHHHHHHHH
MEQVKFWRDKNMAVNPLDFLKNMSSVKNNIDNIKKEISKIT

----------SSSSSSS-----SSSSSSS---------- HHHHHHHH
VCGKAGSNIVTIEMDGEFNVKKVSINKEFFDDLDNDAFEQMI

HHHHHHHHHHHHHHHHHH ----------
KSALNDAVSKVKEEIKLKTMGVLPFGM
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Fig. 1 The secondary structure of BB0462 protein predicted by JUFO program. Note: H represents α-helix, and S represents β-sheet.

3.2 BB0462 protein enhances the activity of *opp*AV promoter

In *B. burgdorferi* oligopeptide permase (Opp), there are five OppA proteins (OppA1–5) encoded by five different *opp*A genes (*opp*AI–V). Each OppA protein is capable of taking oligopeptides from the external environment and transporting it into bacterial cells as the nutrition source *via* Opp transport system (Lin et al., 2001). It has been demonstrated that the upstream region of each *opp*A gene functions as an independent promoter (Wang et al., 2002). To test if BB0462 protein affects activities of the five *opp*A promoters, the recombinant plasmid pET30a/BB0462 was co-transformed into *E. coli* BL21 $(DE)_3$ with different pCB182 recombinant plasmids in which the upstream regulation regions of *opp*AI, II, III, IV or V genes were inserted prior to a *lac*Z gene. Five co-transforming *E. coli* BL21$(DE)_3$ cells, containing pET30a plasmid and the recombinant plasmid pCB182 in which the upstream region of *opp*AI, II, III, IV or V was inserted ahead of the gene *lac*Z, were used as the control. Since pCB182 is a non-promoter plasmid, *lac*Z gene is unable to express if the DNA fragment inserted prior to *lac*Z does not function as a promoter (Schneider and Beck, 1986). Thus, we can know if the DNA inserted functions as a promoter based on β-galactosidase activity. β-galactosidase activities of five cotransformants with or without BB0462 protein in the cells are shown in Fig. 2. β-galactosidase activities in pA1, pA2, pA3 and pA4 did not show any significant difference as compared with their respective controls. In the case of pA5, however, the co-transformed bacterium showed about 50% high activity as compared with its control, which indicated that BB0462 protein enhanced the activity of the *opp*AV promoter.

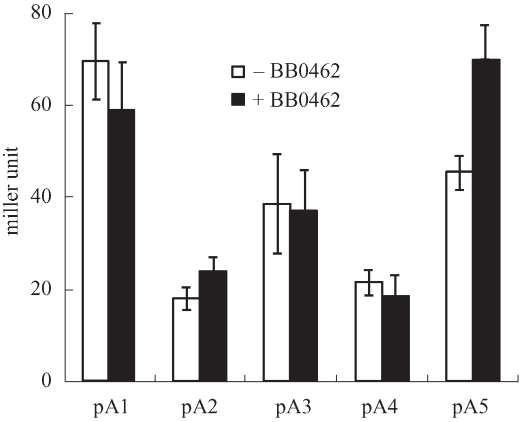

Fig. 2 Analysis of β-galactosidase activity of five different cotransformants containing pET30a/BB0462 and pCB182 recombinant plasmids. Note: PA1–5 represent the recombinant plasmid pCB182 inserted with the corresponding upstream regulation region of *opp*AI, II, III, IV or V; □: the plasmid pET30a without BB0462 gene; ■: the plasmid pET30a/BB0462. Each bar represents the mean of three separate experiments, and error bars represent standard error.

3.3 BB0462 protein binds specifically to the upstream regulation region of *opp*AV

To answer whether the higher activity of the *opp*AV promoter results from the interaction between the BB0462 protein and the upstream regulation region of *opp*AV, the expression of BB0462 protein in five different cotransformants was first examined with the use of Western blotting. In view of the fact that the HA tag (YPYDVPDYA) was fused into the N-terminus of the BB0462 protein, an antibody raised against HA epitope tag was used in this study. The results from Western blotting showed that BB0462 protein presented in all five cotransformant cells except for the control group (data not shown), which ruled out the possibility that no significant increase of the activity of *opp*AI, II, III or IV promoters results from no expression of BB0462 protein in those cells, and which also indicated the possibility that BB0462 protein might interact with the promoter of *opp*AV. To further verify if BB0462 protein interacts with the upstream regulation region of *opp*AV, BB0462 protein was purified with the use of nickel affinity chromatography to homogeneity (Fig. 3). The purified BB0462 protein was mixed respectively with different upstream regulation regions of *opp*AI–V amplified by PCR, and then the gel shift assay was performed. Regardless of using ethidium bromide staining or DIG-labeling detection, the addition of BB0462 protein did not result in any specific retardation of DNA mobility of the upstream regulation region of *opp*AI, II, III, IV or IV on the PAGE gel (data not shown). By contrast, the addition of BB0462 protein resulted in a shift of the upstream DNA fragment of *opp*AV (Fig. 4).

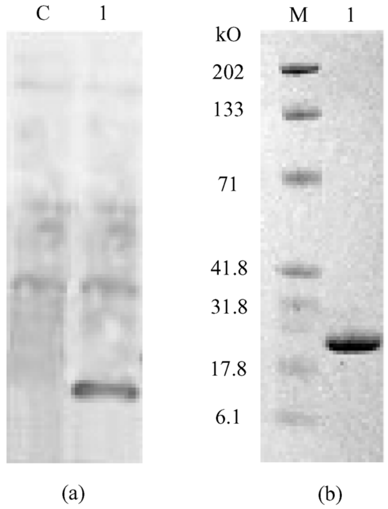

Fig. 3 Expression and purification of BB0462 protein. (a) Western blotting showing the expression of BB0462 protein in *E. coli*. C, the crude extract from *E. coli* BL21 $(DE)_3$ with the plasmid pET30a; 1: the crude extract from *E. coli* BL21 $(DE)_3$ with the plasmid pET30a/BB0462. (b) BB0462 protein purified on 10% SDS-PAGE.

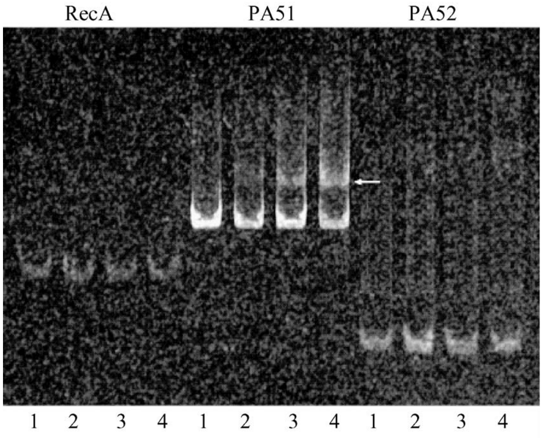

Fig. 4 Gel shift assay with ethidium bromide staining. Note: *rec*A, the 130 bp DNA fragment located in the middle of *E. coli rec*A gene; pA51: the 409 bp upstream regulation fragment close to the initiation codon of *opp*AV gene; pA52: the 92 bp upstream regulation fragment located at the far side of *opp*AV promoter. White-color arrow points the location of DNA retardation on 4% non-denatured polyacrylamide gel. 1,2,3,4 represent 0, 0.5, 1, 2 μL of 80 μg/mL purified BB0462 protein which were added respectively.

OppA5 protein is encoded by *opp*AV gene located in *B. burgdorferi* plasmid lp54 (Johnson et al., 1984), and the upstream region of *opp*AV gene is composed of 501 base pairs. PA52, the 1–92 bp fragment of the *opp*AV upstream region, contains -10 and -35 σ^{70}-type sequences. It was found that PA52 displayed strong promoter activity but its transcriptional direction pointed to another gene rather than *opp*AV (Wang et al., 2002). PA51, the 93–501 bp fragment of *opp*AV upstream region, was also found to represent the promoter activity. Sequence analysis did reveal a putative -10 σ^{70}-like binding sequence located at the position 432, which was close to the initiation codon of *opp*AV (Wang et al., 2002). When BB0462 protein was mixed with PA51, PA52 or *rec*A DNA fragment, it was found that PA51 DNA mobility was retarded with a noticeable dosage effect in the gel shift assay (Figs. 4 and 5), whereas PA52 and *rec*A DNA fragments did not show any shift even if BB0462 protein was used at the highest concentration (Fig. 4). However, with the addition of unlabeled PA51 DNA the mobility-retarded bands vanished (Fig. 5). The result from the competition assay further demonstrated that BB0462 protein did interact specifically with PA51 DNA.

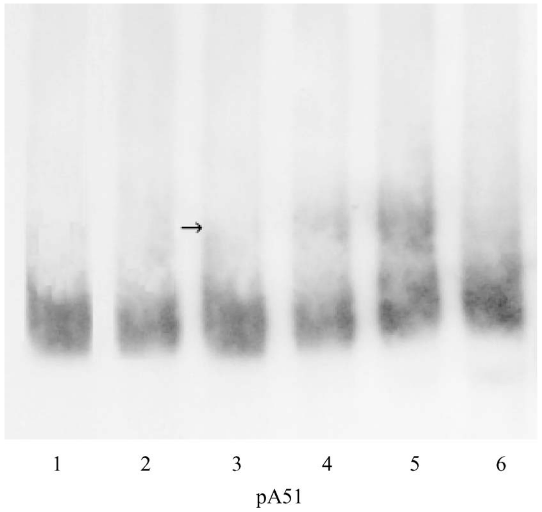

Fig. 5 DIG-labeled gel shift assay showing pA51 DNA retardation and competitive inhibition. In lane 1,3,4,5, 15 fmol DIG-labeled pA51 DNA was mixed with 0, 0.2, 0.5 and 1 μL 6 μM BB0462 protein respectively; In lane 2 and 6, 15 fmol DIG-labeled pA51 DNA was mixed with 1 μL 6 μM BB0462 protein and a 125-fold excess of unlabeled pA51 DNA used as a competitor.

4 Discussion

In this study, we attempted to establish the function of *B. burgdorferi* BB0462 protein. JUFO analysis revealed that the secondary structure of BB0462 protein, similar to those of the YbaB protein family, possessed the structural characteristics of HLH-type transcription activators. The analysis of β-galactosidase activity demonstrated that BB0462 protein did enhance the activity of *opp*AV upstream regulation region, and the gel shift assay further confirmed that BB0462 protein did interact specifically with the PA51 fragment of the *opp*AV upstream regulation region. All of the results in this study suggest that BB0462 protein might be concerned with the regulation of the *opp*AV gene expression. The *opp*AV gene was cloned and expressed in *E. coli*, and the corresponding protein was purified to homogeneity (Wang and Hu, 2005), but the biological function of OppA5 was not fully understood. At present, we only know that the expression of *opp*AV gene *in vivo* is related to the variation of external environments. For instance, previous studies found *opp*AV gene performed a high level of expression when *B. burgdorferi* lived in mice. However, the expression of the *opp*AV gene was quite low when the spirochete lived in tick (Wang et al., 2002). Thus, further investigation is required to fully understand if BB0462 protein is involved in the response of *opp*AV gene to external environments. Actually, a 50% increase of *opp*AV promoter activity suggest that BB0462 protein might be a very weak activator. However, although both *E. coli* and *B. burgdorferi* are gram-positive bacteria, whether the two bacteria share the same mechanism of transcriptional regulation is not clear. In view of the fact that *B. burgdorferi* undergoes an infectious cycle that requires adaptation to different hosts, it is reasonable that *B. burgdorferi* could have more complicated mechanisms of transcriptional regulation in order to adapt to environmental variation. In *B. burgdorferi*, the interaction between BB0462 protein–DNA complex with RNA polymerase may require participation of other regulation proteins. The α-helix located at the C-terminus of the BB0462 protein is composed of 28 amino acids, in which there are 6 acidic amino acids (3 Asp and 3 Glu) making up 21.4%. The 21.4% acidic amino acids in this area are close to the 22.4% acidic amino acids in the transcription-activating domain of the yeast activator GAL4. Nevertheless, as for the protein structure, it is also possible for the α-helix (with 21.4% acidic amino acids) to participate in the formation of homodimers as a dimerization domain. Therefore, it is still needed to further understand the detailed mechanism of interactions between BB0462 protein and *opp*AV promoter. This will help us to better dissect the biological functions of the YbaB protein family.

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