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Verification and dissection of the *ospC* operator by using *flaB* promoter as a reporter in *Borrelia burgdorferi*

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ABSTRACT

The Lyme disease spirochete *Borrelia burgdorferi* must repress expression of outer surface protein C (OspC) to effectively evade specific humoral immunity and to establish persistent infection. This ability largely relies upon a regulatory element, the only operator that has been reported in spirochetal bacteria. Immediately upstream of the *ospC* promoter, two sets of inverted repeats (IRs) constitute small and large palindromes, in which the right IR of the large palindrome contains the left IR of the small one, and may collectively function as the *ospC* operator. In the study, the large palindrome with or without the small IR was fused with an *flaB* promoter, which was used to drive expression of a promoterless *ospC* copy as a reporter gene, and introduced into OspC-deficient *B. burgdorferi*. The presence of the large palindrome alone significantly reduced *ospC* expression driven by the fused *flaB* promoter in the joint tissue of severe combined immunodeficiency (SCID) mice, and rescued spirochetes from elimination by passively transferred OspC antibody in infected SCID mice and specific immune responses elicited in immunocompetent mice, confirming a function of the IRs as an operator. Inclusion of the small IR further enhanced the ability of the large palindrome to reduce the activity of the fused *flaB* promoter, indicating that the small IR is a part of the operator. Taken together, the study led to successful verification and dissection of the *ospC* operator.

1. Introduction

The Lyme disease spirochete, *Borrelia burgdorferi*, is one of the most invasive bacterial pathogens, causing persistent infection despite the development of vigorous immune responses [1,2]. Tight regulation of outer surface protein (Osp) expression is crucial for its pathogenic strategy. The pathogen abundantly expresses OspA/B in the unfed tick [3–6], consistent with an important role of these lipoproteins in spirochetal persistence in the vector [7,8]. A fresh blood meal down-regulates OspA/B and up-regulates OspC and others, a process that prepares *B. burgdorferi* for infection of mammals, regardless of whether OspC is required for salivary gland invasion [9–12]. Repression of OspA/B expression in mammals is critical for the maintenance of the enzootic cycle because their expression would ultimately induce a strong humoral response and, as a result, may effectively block acquisition of *B. burgdorferi* by the vector [13–15], regardless of whether OspA/B can be targeted by borreliacidal antibodies in mammalian tissues [16]. *B. burgdorferi* abundantly expresses OspC only during early infection when the antigen has an important role and before specific humoral responses have developed [17–19]. OspC is not only a strong immunogen but also an effective target of protective immunity; its expression induces a robust humoral response that imposes tremendous pressure on the pathogen [20,21]. To cause persistent infection, *B. burgdorferi* must down-regulate OspC [17,18,22,23]. If *B. burgdorferi* fails to repress OspC expression or undergo escape mutations on the *ospC* gene, infection would be cleared [20]. It is also crucial for *B. burgdorferi* to keep the *ospC* gene off after it is acquired by the tick vector as OspC antibodies in blood meal may kill spirochetes expressing the antigen in the vector [24], leading to discontinuation of the enzootic cycle.

Only three σ factors can be identified from the entire genome of *B. burgdorferi*, including the major factor, RpoD (σ^{70}), and two alternative factors, RpoN (σ^{54}) and RpoS (σ^{38}) [25]. Moreover, the two alternative factors compose a regulatory network, in which RpoS expression depends on RpoN [26,27], greatly limiting their role in contribution to diverse gene regulation. In the unfed tick, the network is silent, so are the RpoS-dependent genes, such as *ospC*, decorin-binding proteins A/B (*dbpA/B*), *ospF* and *bbk32* [3,28–30]. During mammalian infection, *B. burgdorferi* apparently mobilizes all the three σ factors [31–33]; therefore, selective gene down-regulation must depend on mechanisms other than controlling expression of these factors.

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The identification of the *ospC* operator helps to interpret the ability of *B. burgdorferi* to selectively down-regulate *ospC*, while actively transcribing other RpoS-dependent genes during mammalian infection [34]. While the regulatory element remains to be verified in a different system, two sets of inverted repeats (IRs) immediately upstream of the *ospC* promoter constitute small and large palindromes, in which the right IR of the large palindrome contains the left IR of the small one, and may collectively function as an operator but have to be confirmed. Although *ospC* is probably the most investigated among genes in spirochetal bacteria, the full *ospC* promoter region remains to be defined even after attempts from two leading groups in the field [35,36]. In contrast, the *flaB* promoter is a well-defined σ^{70}-dependent promoter, driving constitutive gene expression [37]. In the study, the large palindrome with or without the small right IR was fused with the *flaB* promoter, which was used to drive expression of a promoterless *ospC* copy as a reporter gene, and introduced into OspC-deficient *B. burgdorferi*. The study allowed us to successfully verify and dissect the *ospC* operator.

2. Results

2.1. Identification of a minimum flaB promoter

The efficiency for the *ospC* operator to repress the activity of a downstream promoter is likely position-dependent, so unessential upstream sequences of the *flaB* promoter should be removed. To identify a minimum *flaB* promoter, two constructs containing the identical promoterless *ospC* copy as a reporter gene were used. The construct pBBE22-*flaBp*$_{min}$-*ospC*, which was created as illustrated in Fig. 1A,B, harbored an *flaB* promoter sequence extending just to the −35 region, consequently called a minimum promoter; while pBBE22-*ospC′* constructed in our previous study [20], contained an *flaB* promoter extending to −192 from the transcriptional start site and was expected to drive maximum transcriptional activity. The feature of the constructs was summarized in Table 1.

The two constructs were electroporated into the *ospC* mutant, which was generated and characterized in our previous study [34]; 16 and 18 transformants were obtained from the transformation with each construct. Plasmid analyses identified two clones receiving each construct, namely *Bp-C*/1, *Bp-C*/2, *ΔospC*/*ospC′*/1, and *ΔospC*/*ospC′*/2. The clones *Bp-C*/1 and *Bp-C*/2 received pBBE22-*flaBp*$_{min}$-*OspC*, while the clones *ΔospC*/*ospC′*/1 and *ΔospC*/*ospC′*/2 obtained pBBE22-*ospC′*. These clones shared the same plasmid content as the *ospC* mutant, which lost lp25, lp56, lp5, lp21 and cp9 [34]. OspC expression resulting from introduction of the constructs was confirmed by immunoblot analysis (Fig. 1C), demonstrating that both constructs actively drove *in vitro* OspC expression.

To more precisely compare the activity of the constructs to drive reporter expression, the *Bp-C*/1, *Bp-C*/2, *ΔospC*/*ospC′*/1, and *ΔospC*/*ospC′*/2 spirochetes were harvested at early log and stationary phase. RNA was prepared and analyzed for *ospC* and *flaB* mRNA accumulation by RT-qPCR. At early log phase, introduction of the constructs pBBE22-*ospC′* and pBBE22-*flaBp*$_{min}$-*ospC* led to the accumulation of 2049 and 1926 *ospC* transcripts, respectively, to match every 1000 *flaB* mRNA copies (Fig. 2A), indicating that the two promoter versions initiated reporter expression as equally well ($P = 0.26$). At stationary phase, the minimum *flaB* promoter drove *ospC* transcription also as effectively as the long promoter version ($P = 0.47$); although the two constructs increased *ospC* mRNA copies to 5281 and 5137, respectively, for every 1000 *flaB* transcripts produced, representing 2.6- and 2.7-fold increases over early log phase (*P* values were 5.7×10^{-7} and 6.9×10^{-7}, respectively).

To explore how the constructs more effectively increased *ospC* mRNA accumulation at stationary rather than early log phase, the copy numbers of the *kan* gene on the constructs and the chromosomal *flaB* gene were determined by qPCR. As shown in Fig. 2B, *B. burgdorferi* produced, on average, six copies of the constructs to match each copy of the linear chromosome at early log phase and increased to over 40 copies at stationary phase, representing a nearly 7-fold increase ($P = 7.1 \times 10^{-6}$). Compared to a less than 3-fold increase in *ospC* mRNA (Fig. 2A), the fused *flaB* promoter did

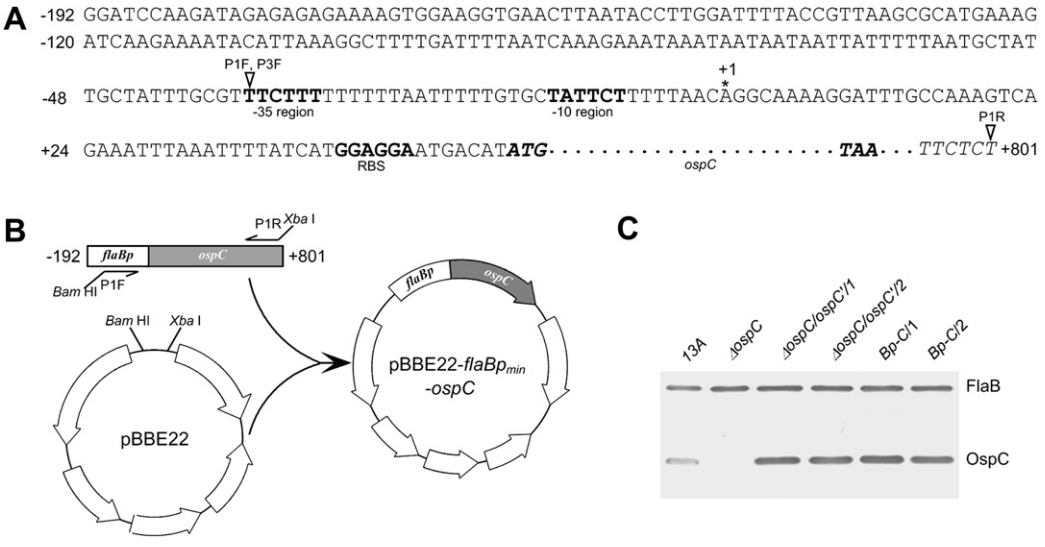

Fig. 1. Identification of a minimum *flaB* promoter. (A) Extended *flaB* promoter sequence and fused promoterless *ospC* gene. This sequence is already in pBBE22-*ospC′*, which was constructed from pBBE22 in our previous study [20]. The sequence includes the *flaB* promoter and upstream sequence, extending to −192 from its transcriptional start site, and a promoterless *ospC* gene (italic), extending from the start codon ATG (boldface) to +801 from the transcriptional start site of the *ospC* gene. The stop codon TAA (boldface) of *ospC* is also present. The −35 and −10 regions, and the putative ribosome-binding site (RBS) (all in boldface type) of the *flaB* gene are indicated. The asterisk marks the previously identified transcriptional initiation site [44]. The amplification start sites of primers, P1F, P3F and P1R, are pointed with open triangles. P1F and P3F had an identical sequence, but were incorporated with different restriction enzyme sites, and were used for plasmid construction described in this figure and Fig. 3, respectively. (B) Construction of pBBE22-*flaBp*$_{min}$-*ospC* from pBBE22 and pBBE22-*ospC′*. An 883-bp fragment was PCR amplified with the use of primers P1F and P1R, and of pBBE22-*ospC′* as a template, then cloned into pBBE22. (C) Immunoblot analysis of OspC expression. The parental clone 13A, the *ospC* mutant (*ΔospC*), and the clones *ΔospC*/*ospC′*/1, *ΔospC*/*ospC′*/2, *Bp-C*/1 and *Bp-C*/2 were verified for OspC expression by immunoblotting probed with a mixture of FlaB and OspC MAbs.