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Contact-dependent regulation of a *Tannerella forsythia* virulence factor, BspA, in biofilms

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Abstract

Tannerella forsythia is one of the periodontal organisms implicated in the development of periodontal diseases. The surface associated and secreted protein, BspA (encoded by the *bspA* gene), of this bacterium is an important virulence factor. The present study was carried out to examine the regulation of the *bspA* gene during biofilm growth and contact stimuli encountered in interbacterial interactions. The expression levels of the *bspA* transcript were determined by real-time RT-PCR approach. The levels of *bspA* transcript were found to be significantly reduced as a result of contact stimulus and in biofilm cells relative to planktonic cells. The results of our study suggest that the likely downregulation of the BspA protein in biofilms and following contact may have implications in pathogenesis as a plausible mechanism of evasion of host immune responses.

Keywords: *Tannerella forsythia*; Biofilms; Contact-dependent regulation

1. Introduction

Tannerella forsythia (formerly *Bacteroides forsythus*) is one of the microbial pathogens recently implicated in the development of periodontal diseases [1–4]. Information on the pathogenicity of this organism, including its virulence factors and their regulation is generally lacking. We previously identified a cell-surface associated as well as a secreted protein, BspA, from this bacterium [5]. The BspA protein belongs to the leucine-rich repeat (LRR) protein family characterized by the presence of leucine-rich repeat motifs. Most proteins belonging to the LRR family are involved in receptor-ligand recognitions via protein–protein interactions [6]. Our studies have shown that the BspA protein is involved in binding to the extracellular matrix protein components fibronectin and fibrinogen [5], coaggregation with other bacteria [7,8] and induction of proinflammatory cytokines from monocytes [9] as well as a chemokine from osteoblasts [10]. The BspA protein is also the target of host immune responses, as patients with *T. forsythia*-associated periodontal disease mount a BspA-specific serum antibody response [5]. Recently, we have also shown that the *T. forsythia*-induced alveolar bone loss in mice requires expression of the BspA protein [11]. Therefore, the BspA protein may represent an important virulence factor of *T. forsythia* with multifunctional activities involved in bacterial pathogenesis. Since subgingival bacteria form biofilms during the development of periodontitis, regulation of virulence factors during biofilm formation is thought to be

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important in the etiology of these diseases. Therefore, we focused our studies on the regulation of the BspA virulence factor of *T. forsythia* in mono- and polymicrobial biofilms.

Environmental sensing by bacteria while attaching to surfaces, during interbacterial interactions and in biofilms is considered to be important for regulation of bacterial gene expression. In this regard, several surface-contact mediated signals that require specific cell surface receptors have been recently recognized in bacteria which modulate the expression of surface factors involved in cell-surface interactions [12–14]. In the above studies, surface-contact has been shown to alter the relative proportions of different outer membrane proteins. The present study was carried out to investigate the effect of contact stimulus encountered in interbacterial interactions and in biofilms of *T. forsythia* and *Fusobacterium nucleatum*, a fusiform bacterium of the oral cavity, on the expression of the *bspA*. *F. nucleatum* has been shown to coaggregate with many of the early and late colonizing bacteria of the oral cavity [15]. Moreover, as not all of the early colonizers coaggregate with the late colonizers, *F. nucleatum* is thought to act as a bridge between the early and late colonizing bacteria [15]. In addition, our recent studies have shown that coaggregation of *F. nucleatum* with *T. forsythia* is mediated via the BspA protein [8].

2. Materials and methods

2.1. Bacterial strains and culture conditions

The following bacteria were used in the present study: *F. nucleatum* ATCC 10953 and *T. forsythia* ATCC 43037. Bacteria were grown anaerobically in brain heart infusion (Difco Laboratories, Detroit, MI, USA) broth containing 5 μg/ml hemin, 0.5 μg/ml menadione, 0.001% *N*-acetyl muramic acid and 5% fetal bovine serum (Life Technologies, Grand Island, NY, USA) as described previously [5].

2.2. Biofilm formation and measurement

Biofilms were studied under static culture conditions on polystyrene surfaces. Briefly, bacterial cultures of *T. forsythia* and *F. nucleatum* were grown in half-strength growth medium to an absorbance of 0.05 at 600 nm. Cells were then dispensed (0.5 ml per well) in triplicate wells of 24-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and incubated anaerobically. For mixed biofilms, each bacterial culture was adjusted to an absorbance of 0.05, dispensed into the wells and incubated as above. After a 2-day incubation, planktonic cells were aspirated and biofilms quantified by crystal violet staining [8]. Total biofilms were calculated by normalizing dye binding (absorbance at 595 nm) to total bacterial growth (biofilm cells + planktonic cells) determined from parallel identical wells by measuring the absorbance at 600 nm. The number of *T. forsythia* cells in biofilms were also estimated by a real-time PCR method. Briefly, the bacterial genomic DNA was isolated from biofilm or planktonic cells using the Puregene DNA isolation kit (Gentra systems, Minneapolis, MN, USA). The real-time PCR was performed using MyiQ single color real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). The reaction mixture (25 μl) for real-time PCR assay contained 2× iQ Supermix (Bio-Rad), 20 pmol of each forward and reverse primers, 10 pmol of fluorescent and quencher labeled probe, and 2.5 μl of extracted DNA. Thermocycling program was 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. A standard curve was plotted using the Ct values for the amplification of DNA extracted from known cfu's of *T. forsythia.* Theoretical cell numbers in each sample were then extrapolated from the Ct values using the standard curve.

2.3. Primers and probes

The sequences of primers and probes used in this study are listed in Table 1. For identification of *T. forsythia*, published information on the primers and probe sequences specific for *T. forsythia* 16S rRNA were utilized [16]. On the other hand, primers and probes specific for *bspA* were designed with the help of the Beacon Designer software (Biosoft International, Palo Alto, CA). Probes for 16S rRNA and *bspA* labeled with the reporter dye 6-carboxyfluorescein at the 5′ end and with the quencher dye 6-carboxytetramethylrodamine at the 3′ end were synthesized commercially (Integrated DNA Technologies, Coralville, IA, USA).

2.4. Quantitative assay for expression of bspA transcript

Total RNA was extracted from bacterial cells using the RNeasy mini kit (QIAGEN, Valencia, CA, USA) as per the manufacture's instructions. Prior to reverse transcriptase (RT) reaction, RNA samples were treated with DNase I (DNA-free, Ambion, Austin, TX, USA) for 1 h at 37 °C to remove any contaminating

Table 1
Oligonucleotide primers and probes

Gene	Oligonucleotide sequence (5′–3′)
T. forsythensis 16S rRNA	Fw: ATTGAAATGTAGACGACGGAGAGT Rev: TTACCTGTTAGCAACTGACAGTCA Probe: AGAGCTTTCTTCCCTTCGGGGCGT
T. forsythensis bsp A	Fw: CGAAGCGAAGGACGTATGGAA Rev: GATTGGTGATGGTGAGGGTTTTG Probe: CGGCACGATGGAGAAACGCAACCT