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Regulation of *Streptococcus gordonii sspB* by the *sspA* Gene Product

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Streptococcus gordonii **expresses two related adhesins, SspA and SspB, the genes for which are adjacent on the chromosome and are regulated independently. Although the adhesins are functionally similar, the *sspA* promoter is more active than that of *sspB*. In this study we show an additional role for SspA in the control of *sspB* activity. Gel shift and DNA footprinting assays demonstrate that the SspA protein binds to the *sspB* promoter and protects a region 233 to 264 bp upstream of the predicted −35 promoter element. The responsiveness of the *sspB* promoter to SspA was investigated with a promoter-*cat* reporter. Expression of the *sspB* promoter was reduced by over 60% in an SspA-deficient mutant of *S. gordonii*. These results indicate that expression of *S. gordonii sspB* is positively regulated by the *sspA* gene product.**

Streptococcus gordonii is a prominent colonizer of dental plaque, a microbial biofilm that is strongly associated with the development of caries and periodontal diseases. *S. gordonii* exhibits a wide range of adherence properties that are well characterized at the molecular level (8). Ssp cell surface proteins are multifunctional adhesins that participate in binding reactions with salivary agglutinins and with other plaque bacteria such as *Porphyromonas gingivalis*, an aggressive periodontal pathogen (1, 2, 7). Such interbacterial adhesive interactions are important in the development of plaque and in its transition from a benign accumulation to a potentially pathogenic entity.

In strains of *S. gordonii* thus far examined, tandem genes encode SspA and SspB polypeptides that are highly similar with respect to structure and adhesive function (2, 6). However, the *sspA* and *sspB* genes possess individual promoter regions and are differentially regulated in response to environmental conditions (4). Further, as measured by promoter-*cat* reporter constructs, transcriptional activity of the *sspA* promoter is about threefold higher than that of the *sspB* promoter over a range of growth conditions (4). SspA and SspB may thus have distinct roles to play for the organism. In this study we investigated regulation of the *sspB* gene by the *sspA* gene product.

SspA binds to the *sspB* promoter region. To determine whether the SspA polypeptide can bind to the *sspB* upstream region, a gel mobility shift assay was performed using the BandShift kit (Amersham Pharmacia Biotech). Recombinant SspA protein was expressed from the *sspA* gene and regulatory sequences (2) in *Escherichia coli* DH5α cultured in Luria-Bertani broth. Crude periplasmic preparations were generated by osmotic shock (5). The SspA polypeptide was further purified by Sepharose 6B (Pharmacia) and DEAE-Sephadex (Sigma) chromatography. Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and a single band of SspA protein was detected following silver staining. The region −347 to −17 bp from the *sspB* translational start site (3) was amplified by PCR and cloned into pCRII (TOPO vector; Invitrogen). A 364-bp double-stranded *Eco*RI fragment containing the *sspB* promoter region was used for gel shift analysis. DNA (5 ng) was 5′ labeled with α-^{32}P and purified on an Atlas Nucleospin column (Clontech). Binding reactions of SspA (4 μg of protein) and target DNA were carried out in binding buffer (40 mM Tris-HCl [pH 7.5], 200 mM NaCl, 2 mM dithiothreitol, 50% glycerol, 1% Nonidet P-40) and mixed with a synthetic competitor poly(dI-dC) in a total of 20 μl and then incubated at room temperature for 20 min. Samples were analyzed on a 5% polyacrylamide gel (run in low-ionic-strength gel buffer: 70 mM Tris-HCl [pH 7.5], 30 mM sodium acetate, 10 mM EDTA) for 90 min at 10 V/cm at 4°C. As shown in Fig. 1, purified SspA protein was capable of binding to and retarding the mobility of the *sspB* upstream region. SspA-DNA complex formation was not affected by *E. coli* DNA (0.1 mg/ml), indicating specificity of binding (Fig. 1, lane 4). In contrast, the presence of excess unlabeled target DNA abolished the SspA-mediated gel shift (Fig. 1, lane 3). Additional evidence of specificity was provided by the failure of an *E. coli* extract containing an irrelevant protein (EBNA-1) to shift the target DNA (Fig. 1, lane 5).

SspA binding protects a 32-bp region of the *sspB* promoter. To identify the SspA binding site upstream of the *sspB* gene, DNase I footprinting was performed. As preliminary results indicated that the protected region was at the 5′ end of the region used for gel shift analysis, a 5′ extended target was used for footprinting. A 279-bp upstream *Eco*RI-*Xma*I *sspB* DNA fragment (−418 to −139 from the translational start site) was amplified by PCR and mixed with purified SspA protein in binding buffer (described above) containing 10 mM $MgCl_2$. Digestion was initiated by the addition of 2 U of DNase I (Amersham Pharmacia Biotech). The reactions were stopped by the addition of diluted DNase stop solution (768 mM sodium acetate, 128 mM EDTA, 0.56% sodium dodecyl sulfate, 256 μg of yeast RNA/ml) (Amersham Pharmacia Biotech), and the mixtures were extracted with an equal volume of phenol-chloroform (1:1). The nucleic acids were then precipitated,

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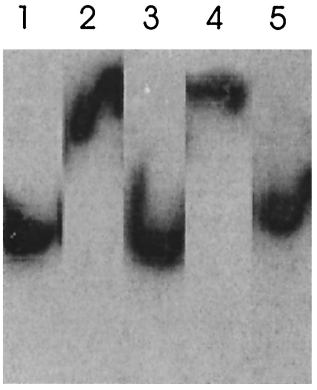

FIG. 1. Electrophoretic mobility shift analysis of binding of SspA to the *sspB* upstream promoter-containing region. A 364-bp ^{32}P-labeled *sspB* upstream fragment was used as the probe. Reaction mixtures contained probe alone (lane 1), probe with SspA protein (lane 2), probe with SspA and excess unlabeled probe (lane 3), probe with SspA protein and excess *E. coli* DNA (lane 4), or probe with sonicated *E. coli* extract containing cloned DNA binding domain from EBNA-1 (lane 5).

dried, resuspended in loading buffer (deionized formamide containing 10 mM EDTA, 0.3% bromophenol blue, and 0.3% xylene cyanol), and electrophoresed in 8% polyacrylamide gels containing 7 M urea. The Maxam and Gilbert A+G sequencing ladder (9) was used as a size standard for the DNase I protection experiments. The results revealed that a 32-bp region of the *sspB* upstream sequence is fully protected from DNase I digestion in the presence of SspA (Fig. 2). This protected sequence spans −342 to −311 bp from the translational start site of the *sspB* promoter and is 233 to 264 bp upstream of the predicted −35 promoter element.

Disruption of the *sspA* gene affects *sspB* expression. To determine whether the binding of SspA to the *sspB* upstream region influences transcription of *sspB* in *S. gordonii*, an *sspB* promoter-*cat86* reporter fusion was introduced into the genome of an SspA-deficient strain of *S. gordonii. S. gordonii* strains were cultured aerobically in Trypticase peptone broth supplemented with yeast extract (5 mg/ml) and 0.5% glucose at 37°C. *S. gordonii* OB220 (kindly provided by Howard Jenkinson, University of Bristol) is derived from strain DL1 and contains an insertional inactivation of the *sspA* gene (2). Recombinant strain HA00 was described previously (4) and is a derivative of DL1 containing a chromosomal *sspB* promoter-*cat* reporter fusion. Strain HA02 was constructed by a strategy similar to that for HA00 and is a derivative of OB220 (SspA deficient) containing a chromosomal *sspB* promoter-*cat* reporter fusion. Briefly, suicide plasmid pHA145 (4), which encodes tetracycline resistance and contains a 1.1-kb *Bam*HI fragment with the *S. gordonii sspB* promoter-*cat* fusion, was isolated by Wizard Miniprep (Promega) and integrated into the chromosome of OB220. Transformants resulting from a single (Campbell) crossover were selected on brain heart infusion agar containing tetracycline (10 μg/ml).

To confirm the integration events, nylon blots of *Hin*dIII- or *Eco*RI-digested chromosomal DNA were probed with biotin-labeled *sspB* promoter or the *cat* gene (not shown). In addition, the inserted fragment was amplified by PCR and sequenced directly using a *cat* defined primer (5′-CAGGAGTCCAAAT ACCAGAGAAT-3′). Hence, the recombinant strains HA00 (*sspB*$_p$:*cat*) and HA02 (SspA-deficient *sspB*$_p$:*cat*) contain two copies of the *sspB* promoter, one driving expression of CAT (chloramphenicol acetyltransferase) and the other driving expression of the structural SspB protein. Strains HA00 and HA02 were grown in batch culture, and CAT activity was measured over time. Cells were harvested by centrifugation (6,000 × *g*, 10 min, 4°C) and washed once in TPE buffer (100 mM Tris-HCl [pH 7.8], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Cells were then suspended in spheroplasting buffer (20 mM Tris-HCl [pH 6.8], 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 2% raffinose, 500 U of mutanolysin/ml) and incubated at 37°C for 30 min. To disrupt the cells, 0.1-ml glass beads (0.10 to 0.11 mm in diameter; Braun Melsugen AG) and 0.4 ml of ice-cold TPE buffer were added and the cells were vortex mixed twice for 30 s. Suspensions were then centrifuged (12,000 × *g*, 20 min, 4°C) to pellet beads and cell debris, and supernatants were removed for enzyme assays. Protein concentrations were determined by a Bio-Rad protein assay kit with bovine gamma globulin as the standard. CAT enzyme activity was assayed by the spectrophotometric method of Shaw (10), utilizing a Beckman DU-70 recording spectrophotometer with a temperature-controlled cuvette chamber. Briefly, 0.05 ml of streptococcal cell extract (containing 40 to 60 μg of protein) was added to a 0.5-ml reaction mixture (100 mM Tris-HCl [pH 7.8], 0.1 mM acetyl coenzyme A [Sigma], 1 mM 5,5′-dithiobis-2-nitrobenzoic acid) in 1-ml glass cuvettes. The reaction mixture was warmed at 37°C for 2 min, and the background change in absorbance at 412 nm was recorded for a further 2 min. Chloramphenicol (16 μl, 0.1 mM) was added, and the change in absorbance was recorded for 2 min. The reaction rate was determined from the linear portion of the graph, corrected for the background change in A_{412}, and divided by 0.0136 to yield CAT activity expressed as nanomoles of chloramphenicol acetylated per minute at 37°C.

As shown in Fig. 3, *sspB* promoter activity, as determined by CAT specific activity, was reduced by 60 to 95% over the growth curve in strain HA02 (SspA deficient) compared to

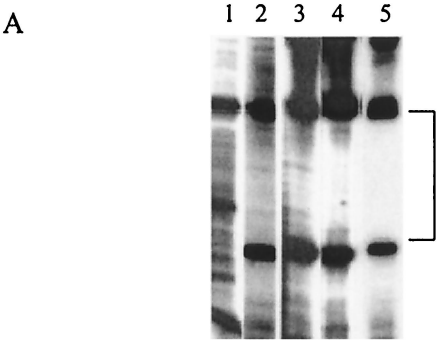

FIG. 2. (A) DNase I footprint of protection by SspA of a 279-bp *sspB* upstream region. Lane 1, Maxam and Gilbert A+G ladder; lanes 2 to 5, DNase I treatment in the presence of 0, 1, 2, and 4 μg of SspA, respectively. The 32-bp protected sequence is indicated by a bracket. (B) DNA sequence upstream of *sspB*. The 32-bp region protected from DNase I digestion is underlined.

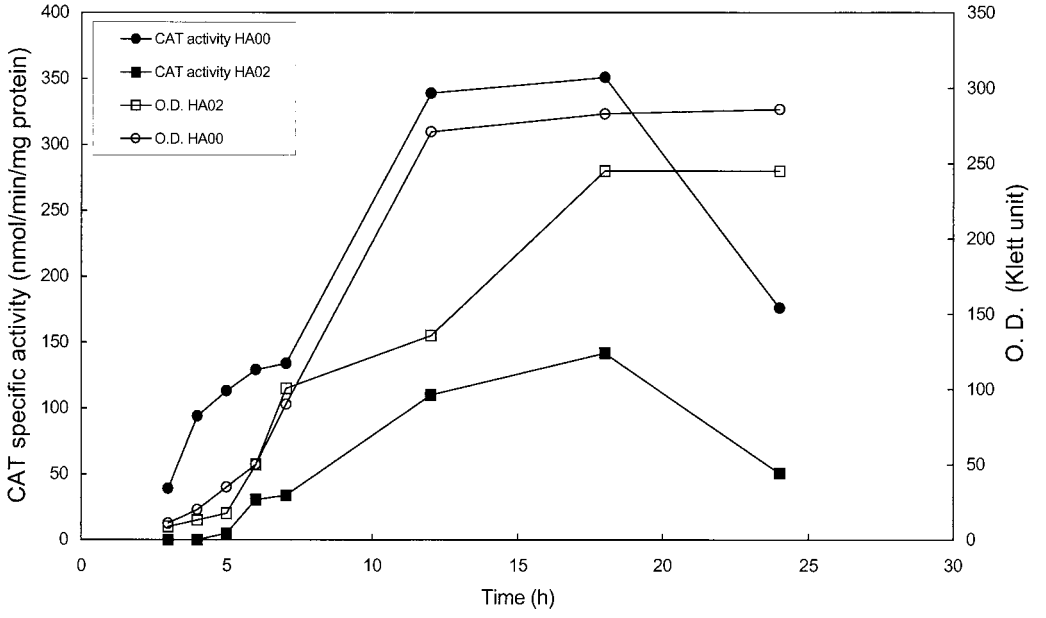

FIG. 3. Growth phase and expression of *sspB* in an SspA$^+$ (strain HA00) or SspA$^-$ (strain HA02) background. Optical densities (O.D.) and CAT activities were measured at different incubation times. The data are from a representative of three experiments.

HA00 (SspA$^+$). In both strains, *sspB* promoter activity was lowest in the lag phase and increased 10-fold in late-log-phase cells, as observed previously (4), indicating that growth phase-dependent regulation of *sspB* is independent of SspA and was not disrupted by the chromosomal insertions. Growth curves of HA00 and of HA02 were similar, although HA02 grew slower and to a slightly lower final density. However, previous results (4) demonstrated that *sspB* promoter-driven *cat* expression is not affected by doubling time. Thus, the reduction in *sspB* expression in HA02 is unlikely to be a reflection of the reduced growth rate of the organism. The results are therefore consistent with positive regulation of *sspB* by the *sspA* gene product. It is possible that the presence of *ermAM*, which was used to disrupt *sspA* (2), affects transcription of *sspB*. However, *sspA* and *sspB* are independently transcribed, and a stem-loop structure that resembles a transcription terminator exists just downstream of *sspA* (3, 4, 7), thus arguing against this situation. Furthermore, expression of SspB occurs in the *sspA* mutant strain (2) and other control pathways are not disrupted in HA02, as evidenced by the response of the *sspB* promoter to the growth phase of the organism.

The advantages to *S. gordonii* conferred by the production of two structurally and functionally related adhesins (SspA and SspB) can be hypothesized to include more avid attachment, diversity of substrate recognition, and immune avoidance. This arrangement, however, may necessitate tightly controlled expression of the genes. Our previous studies have shown that the *sspA* and *sspB* genes each possess functional promoters with differing activities that respond independently to environmental cues (4). The results of this study indicate that in addition to its role as a surface adhesin, intracellular SspA can positively regulate transcription of the *sspB* gene through binding to a region 233 to 264 bp upstream of the *sspB* −35 site. Thus, although the transcriptional activities of *sspA* and *sspB* differ according to prevailing conditions in the oral cavity, expression of SspA and SspB is linked through the activity of the SspA protein.

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