Role of the *Streptococcus gordonii* SspB protein in the development of *Porphyromonas gingivalis* biofilms on streptococcal substrates

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***Porphyromonas gingivalis* is an aggressive periodontal pathogen that persists in the mixed-species plaque biofilm on tooth surfaces. *P. gingivalis* cells attach to the plaque commensal *Streptococcus gordonii* and this coadhesion event leads to the development of *P. gingivalis* biofilms. Binding of these organisms is multimodal, involving both the *P. gingivalis* major fimbrial FimA protein and the species-specific interaction of the minor fimbrial Mfa1 protein with the streptococcal SspB protein. This study examined the contribution of the Mfa1–SspB interaction to *P. gingivalis* biofilm formation. *P. gingivalis* biofilms readily formed on substrata of *S. gordonii* DL1 but not on *Streptococcus mutans* cells which lack a coadhesion-mediating homologue of SspB. An insertional inactivation of the *mfa1* gene in *P. gingivalis* resulted in a phenotype deficient in *S. gordonii* binding and unable to form biofilms. Furthermore, analysis using recombinant streptococci and enterococci showed that *P. gingivalis* biofilms formed on *Enterococcus faecalis* strains expressing SspB or translational fusions of SspB with SpaP (the non-adherent SspB homologue in *S. mutans*) containing the *P. gingivalis* adherence domain (SspB adherence region, BAR) of SspB. In contrast, an isogenic Ssp null mutant of *S. gordonii* DL1 was unable to support biofilm growth, even though this strain bound to *P. gingivalis* FimA at levels similar to wild-type *S. gordonii* DL1. Finally, site-specific mutation of two functional amino acid residues in BAR resulted in SspB polypeptides that did not promote the development of *P. gingivalis* biofilms. These results suggest that the induction of *P. gingivalis* biofilms on a streptococcal substrate requires functional SspB–minor fimbriae interactions.**

Keywords: minor fimbriae, antigen I/II, coadhesion

INTRODUCTION

Dental plaque is a complex and dynamic biofilm that accumulates through the sequential and ordered colonization of over 500 different species of bacteria (Kolenbrander & London, 1993; Marsh, 1994; Rosan & Lamont, 2000). Plaque that comprises predominantly Gram-positive commensals such as *Streptococcus gordonii* and the related sanguis group of streptococci, and *Actinomyces* spp., can exist in the oral cavity in the

absence of overt disease (Marsh, 1989, 1994; Rosan & Lamont, 2000). However, population shifts within the plaque community that lead to over-representation of acidophiles or of Gram-negative anaerobes may contribute to the initiation and progression of the most common human oral diseases, caries and periodontal disease. Adult periodontitis is associated with subgingival plaque containing elevated levels of specific Gram-negative anaerobic bacteria, including *Porphyromonas gingivalis* (Socransky & Haffajee, 1992). In addition to its niche in the subgingival biofilm, *P. gingivalis* has also been shown to invade gingival epithelial cells (Belton *et al.*, 1999; Lamont *et al.*, 1995) and to possess numerous virulence factors (e.g. pro-

Abbreviations: BAR, SspB adherence region; CSLM, confocal scanning laser microscopy.

teases) that could contribute to periodontal tissue destruction and alveolar bone resorption (Curtis *et al.*, 1999; Lamont & Jenkinson, 1998, 2000; Travis *et al.*, 2000).

Colonization of the plaque biofilm by *P. gingivalis* is contingent upon a variety of factors, including reduced oxygen tension, sufficient nutritional resources and an appropriate substrate for adhesion (Bradshaw *et al.*, 1998; Lamont & Jenkinson, 2000; Marsh, 1989, 1994). Furthermore, as a secondary colonizer of plaque, *P. gingivalis* is likely to encounter tissue surfaces that are already colonized with antecedent bacteria and their products. Consistent with these constraints, *P. gingivalis* has been shown to adhere to primary colonizing organisms such as *S. gordonii* and related streptococcal species, *Actinomyces naeslundii*, and to other Gram-negative secondary colonizing organisms such as *Fusobacterium nucleatum*, *Treponema denticola* and *Bacteroides forsythus* (Kinder & Holt, 1989; Kolenbrander & Andersen, 1989; Lamont *et al.*, 1992; Schwarz *et al.*, 1987; Yao *et al.*, 1996). Coadhesion of *P. gingivalis* with these organisms involves specific adhesin–receptor interactions. For example, a galactose-specific lectin mediates adherence of *F. nucleatum* to *P. gingivalis* (Shanitzki *et al.*, 1997). The coadhesion of *P. gingivalis* and *S. gordonii* is multimodal and involves at least two distinct adhesin–receptor pairs. The major and minor fimbriae of *P. gingivalis* both have been shown to bind to *S. gordonii* cells (Chung *et al.*, 2000; Lamont *et al.*, 1993). The major fimbriae, which may extend up to 3 µm from the cell surface, are composed of the FimA protein (Yoshimura *et al.*, 1984) and interact with a component of *S. gordonii* that has not yet been identified. In contrast, the minor fimbriae extend only 0.1–0.5 µm from the cell surface (Hamada *et al.*, 1996) and contribute to coadhesion by binding to the Ssp polypeptides (Brooks *et al.*, 1997; Demuth *et al.*, 2001). FimA and the minor fimbrial protein (designated Mfa1) are genetically and antigenically distinct (Arai *et al.*, 2000), suggesting that these two adhesins differ both structurally and functionally.

The Ssp proteins are members of the antigen I/II family of streptococcal surface proteins that are highly conserved in overall structure and primary sequence across all the human oral streptococcal species (Jenkinson & Demuth, 1997). However, despite the high degree of structural similarity, *P. gingivalis* adheres to streptococci and interacts with antigen I/II proteins in a species-specific manner. Our previous studies have shown that *P. gingivalis* adheres avidly to the SspA and SspB polypeptides of *S. gordonii*, but does not interact with the SpaP protein, the homologue of SspA/B expressed by *Streptococcus mutans* (Brooks *et al.*, 1997). Adherence is mediated by a discrete domain, designated BAR (SspB adherence region), comprising amino acid residues 1167–1250 of SspB, which is fully conserved between SspB and SspA (Brooks *et al.*, 1997; Demuth *et al.*, 2001). Within BAR, Asn¹¹⁸² and Val¹¹⁸⁵ have been suggested to confer a unique secondary structural motif that is recognized and bound by the *P. gingivalis* minor

fimbrial protein (Demuth *et al.*, 2001). This structural motif (and Asn¹¹⁸² and Val¹¹⁸⁵) is essential for adherence and is not conserved in SpaP polypeptide.

Using an open flow chamber under conditions of low shear force, Cook *et al.* (1998) showed that subsequent to its adherence to *S. gordonii*, *P. gingivalis* rapidly accretes to form a biofilm consisting of structures resembling towering microcolonies separated by fluid-filled channels. Although adherence is clearly a first step that is necessary for accretion to occur, it is not known how the two independent fimbrial-mediated adherence mechanisms of *P. gingivalis* contribute to biofilm formation. In this study, we show that the formation of *P. gingivalis* biofilms exhibits the same species specificity observed in minor-fimbriae-mediated adherence, suggesting that the Mfa1-mediated interaction of *P. gingivalis* with the streptococcal Ssp polypeptides may drive biofilm development. Moreover, *P. gingivalis* biofilms do not form on an Ssp null mutant of *S. gordonii* which retains the ability to interact with FimA, or when the *mfa1* gene of *P. gingivalis* has been disrupted. In addition, recombinant *Enterococcus faecalis* strains expressing chimeric SspB/SpaP proteins containing BAR support biofilm growth, whereas hybrid proteins without BAR do not. SspB proteins containing site-specific mutations of essential functional amino acid residues of BAR do not permit *P. gingivalis* biofilm development. These results suggest that the interaction of the minor fimbriae with Ssp is sufficient to allow *P. gingivalis* to form biofilms on a streptococcal substrate.

METHODS

Bacteria and culture conditions. Bacterial strains used in this study are listed in Table 1. *P. gingivalis* strains were cultured in Trypticase soy broth (BBL) supplemented with 1 mg yeast extract ml⁻¹, 5 µg haemin ml⁻¹ and 1 µg menadione ml⁻¹, under anaerobic conditions (85% N₂, 10% H₂, 5% CO₂) at 37 °C overnight. Erythromycin at 10 µg ml⁻¹ was added to the medium when necessary. For radio-labelling, [³H]thymidine was added to the culture medium at 3.7 × 10⁵ Bq ml⁻¹. Streptococci and enterococci were grown in Trypticase peptone broth (BBL) supplemented with 5 mg yeast extract ml⁻¹ and 0.5% glucose as carbon source, at 37 °C under static conditions. *S. gordonii* OB219 is a derivative of strain DL1 in which both the *sspA* and *sspB* genes are insertionally inactivated (Demuth *et al.*, 1996; Jenkinson *et al.*, 1993) and was a kind gift from Dr Howard Jenkinson, University of Bristol.

Construction of recombinant *E. faecalis* strains. *E. faecalis* SspB expresses full-length SspB peptide encoded by the *S. gordonii* *sspB* gene (Demuth *et al.*, 1989). *E. faecalis* strains Spac4 and Spac5 express the hybrid SspB/SpaP peptides Spac4 and Spac5 derived from a fusion of specific portions of the *S. gordonii* *sspB* and the *S. mutans* *spaP* genes. Construction of these strains has been reported previously (Demuth *et al.*, 2001). As shown schematically in Fig. 1, Spac5 differs from Spac4 only in that it contains the portion of SspB representing BAR. *E. faecalis* NG expresses full-length SspB protein that possesses a glycine substitution for asparagine at position 1182 in BAR. Similarly, *E. faecalis* VP expresses full-length SspB

Table 1. Bacterial strains

Strain	Relevant characteristics*	Source or reference
<i>P. gingivalis</i> 33277	Type strain from ATCC	Laboratory stock
<i>P. gingivalis</i> SMF1	Derivative of 33277 with an insertional inactivation of the <i>mfa1</i> gene; Em ^R	This study
<i>S. gordonii</i> DL1	Wild-type	Laboratory stock
<i>S. gordonii</i> OB219	Derivative of DL1, <i>sspAB::ermAM</i> ; Em ^R	Demuth <i>et al.</i> (1996)
<i>S. mutans</i> KPSK2	Clinical isolate	Laboratory stock
<i>E. faecalis</i> S161	Wild-type	Laboratory stock
<i>E. faecalis</i> 401	Derivative of S161 containing pAM401; Cm ^R	Demuth <i>et al.</i> (1989)
<i>E. faecalis</i> SspB	Derivative of S161 expressing <i>S. gordonii</i> SspB; Cm ^R	Demuth <i>et al.</i> (1989)
<i>E. faecalis</i> Spac4	Derivative of S161 expressing hybrid SspB/SpaP polypeptide Spac4; Cm ^R	Demuth <i>et al.</i> (2001)
<i>E. faecalis</i> Spac5	Derivative of S161 expressing hybrid SspB/SpaP polypeptide Spac5; Cm ^R	Demuth <i>et al.</i> (2001)
<i>E. faecalis</i> NG	Derivative of S161 expressing SspB containing N/G ¹¹⁸² substitution; Cm ^R	Demuth <i>et al.</i> (2001)
<i>E. faecalis</i> VP	Derivative of S161 expressing SspB containing V/P ¹¹⁸⁵ substitution; Cm ^R	Demuth <i>et al.</i> (2001)
<i>E. faecalis</i> DM	Derivative of S161 expressing SspB containing both N/G ¹¹⁸² and V/P ¹¹⁸⁵ substitutions; Cm ^R	Demuth <i>et al.</i> (2001)

* ATCC, American Type Culture Collection, Manassas, VA, USA; Em^R, Cm^R, resistance to erythromycin and chloramphenicol respectively.

Fig. 1. Structure of antigen-I/II-related polypeptides used in this study. SspB from *S. gordonii* contains the BAR domain, encompassing residues 1167–1250, which mediates the *in vitro* adhesion of *P. gingivalis* cells to *S. gordonii*. Spac4 comprises SspB residues 1–1166 (black) fused to residues 1167–1500 of SpaP (grey). Spac5 comprises residues 1–1250 of SspB (black) fused to residues 1251–1500 of SpaP (grey). NG, VP and DM are derived from SspB and contain the denoted amino acid substitutions within BAR. Conserved alanine-rich and proline-rich repetitive domains of SspB and SpaP are labelled HR and PR, respectively.

containing a proline residue replacing the native valine at position 1185 in BAR, and *E. faecalis* DM expresses SspB containing both Asn/Gly¹¹⁸² and Val/Pro¹¹⁸⁵ substitutions (Fig. 1). Site-specific mutations were introduced into *sspB* using the QuikChange Site Directed Mutagenesis Kit (Stratagene) and the appropriate forward and reverse oligonucleotide primers. Conditions for the generation of the specific mutations in the *sspB* gene and the sequences of the oligonucleotide primers were described previously (Demuth *et al.*, 2001). All gene constructs were transferred into the *E. faecalis*–*Escherichia coli* shuttle vector, pAM401 (Wirth *et al.*, 1986) and transformed into *E. faecalis* S161 by electroporation. *E. faecalis* S161 does not express an endogenous homologue of antigen I/II. Recombinant colonies were selected on the appropriate agar plates containing 15 µg chloramphenicol

ml⁻¹. Chimeric gene constructs were confirmed by restriction mapping. The site-specific mutations were confirmed by nucleotide sequencing at the University of Pennsylvania Automated Sequencing Facility.

ELISA of fimbriillin binding. Purified recombinant fimbriillin (FimA) was produced from the *P. gingivalis* *fimA* gene in the pET30 expression system as described previously (Xie *et al.*, 2000). For ELISA experiments, *S. gordonii* DL1 or OB219 cells (10⁷) were deposited onto microtitre plate wells. After blocking with 3% BSA and washing, bacterial cells were reacted (1 h, 37 °C) sequentially with varying concentrations of FimA, specific FimA antibodies (Xie *et al.*, 2000), diluted 1:10000, and alkaline phosphatase-conjugated anti-rabbit IgG. Binding reactions were detected with *p*-nitrophenyl phosphate and A₄₀₅ was recorded. Controls without strepto-

coccal cells or without FimA were included in all experiments and were less than 10% of specific values.

Construction of *P. gingivalis* minor-fimbriae-deficient mutant. *P. gingivalis* SMF1 was created by homologous recombination between *P. gingivalis* 33277 chromosomal DNA and a suicide plasmid carrying an internal fragment of the *mfa1* gene as follows. A 0.85 kb internal fragment of *mfa1* was amplified by PCR with the primers 5'-TATCCGAGGC-CAATGCTATC-3' and 5'-GCATCAAGAAGTTGGGCTTC-3'. Amplification conditions were denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 3 min, for 30 cycles. The product was cloned into the PCR cloning vector pCR11 (Invitrogen). A *Bam*HI-*Xba*I fragment was excised and ligated into the shuttle vector pVA3000 that contains the antibiotic resistance cassette *ermAM-ermF* (Lee *et al.*, 1996). The resulting plasmid was conjugated into *P. gingivalis* 33277 as described previously (Park & Lamont, 1998). Transconjugants with erythromycin resistance were selected. Confirmation that the plasmid had integrated into the *mfa1* gene was achieved by Southern blotting and loss of mRNA expression was established by RT-PCR using the primers defined above.

Interbacterial binding assay. Coadhesion between *P. gingivalis* and *S. gordonii* was measured with the nitrocellulose blot assay described previously (Lamont *et al.*, 1992). Briefly, *S. gordonii* cells were suspended in buffered KCl (5 mM KCl, 2 mM K₂PO₄, 1 mM CaCl₂, pH 6.0) and 10⁸ bacteria were deposited on nitrocellulose paper in a dot-blot apparatus. The blot was washed three times in KCl containing 0.1% Tween 20 (KCl-Tween). The adsorbed bacteria were subsequently incubated for 2 h at room temperature with [³H]thymidine-labelled *P. gingivalis* strains 33277 or SMF1 (mean c.p.m. per cell, 5 × 10⁻⁴) suspended in KCl-Tween. After washing to remove unbound organisms, the experimental areas of the nitrocellulose were excised and adherence was quantified by scintillation spectroscopy.

Biofilm formation. Biofilm studies were carried out essentially as described by Cook *et al.* (1998). Briefly, standard glass coverslips were coated with centrifugation-clarified pooled human saliva for 30 min at 37 °C and washed three times with sterile PBS. Saliva-coated glass coverslips were mounted under aseptic conditions in a polycarbonate flow cell (0.6 × 1.0 cm) with an attached peristaltic pump. Flow cells were inoculated initially with the streptococcal or enterococcal strains (10⁷ cells ml⁻¹) at a flow rate of 4.1 ml h⁻¹ for 2 h (*S. gordonii*) or 4 h (*S. mutans* and *E. faecalis*). *S. mutans* and *E. faecalis* adhere less efficiently to saliva surfaces in comparison to *S. gordonii*, hence the longer incubation period. The formation of a 70–80% confluent bacterial monolayer was confirmed by phase-contrast microscopy. The flow cells were then subjected to a secondary inoculation of *P. gingivalis* (10⁷ cells ml⁻¹) under similar flow conditions. The total length of exposure of streptococci or enterococci to *P. gingivalis* was 4 h, which is less than the doubling time for *P. gingivalis* cells when grown in rich broth. Thus, under these conditions the accumulation of *P. gingivalis* biofilms is a result of accretion of planktonic cells rather than growth of bound cells. Biofilms were visualized with a Bio-Rad MRC600 confocal scanning laser microscope with an Olympus IMT-2 inverted light microscope and a MS plan 60 × 1.4 NA objective. For confocal scanning laser microscopy (CSLM), streptococci and enterococci were stained with 10 mg hexidium iodide ml⁻¹ and *P. gingivalis* was stained with 10 mg fluorescein ml⁻¹. CSLM was first used in the reflected white light mode to directly observe

biofilm formation over the course of the experiments. A representative area of the coverslip was then selected and observed under reflected laser light of 488, 546 and 647 nm. A series of fluorescent optical sections were collected to determine the depth of the bacterial layers and/or microcolonies and to assemble a three-dimensional view of the biofilms using the Slicer (Fortner Research) imaging program. Under these conditions of biofilm formation, *P. gingivalis* cells were observed to gradually and continuously accumulate on the substratum over the 4 h inoculation period. Sloughing and reattachment of biofilm microcolonies was not observed and no significant auto-aggregation of planktonic *P. gingivalis* cells occurred.

RESULTS

Species specificity of *P. gingivalis* biofilm formation

The adherence of *P. gingivalis* minor fimbriae with streptococci is specific for the antigen I/II proteins (Ssp) expressed by *S. gordonii* and does not occur with related antigen I/II polypeptides (SpaP) of *S. mutans* cells (Brooks *et al.*, 1997). To determine if biofilm formation exhibits a similar species specificity, we tested the ability of *P. gingivalis* to accumulate on substrata of *S. gordonii* or *S. mutans* attached to saliva-coated glass coverslips in a flow cell. Planktonic *P. gingivalis* cells adhered rapidly to the *S. gordonii* DL1 substratum and accumulated progressively over the 4 h inoculation period to form large towering microcolonies (Fig. 2a). In contrast, no accumulation occurred on a substratum composed of *S. mutans* KPSK2 cells (Fig. 2b) or with an isogenic mutant of *S. gordonii* DL1 (strain OB219) that does not express antigen I/II (Fig. 2c). The *P. gingivalis* microcolonies that formed on *S. gordonii* DL1 routinely accumulated to depths of 60–80 µm, whereas the streptococcal substratum in the absence of *P. gingivalis* cells was approximately 5–10 µm in depth. *P. gingivalis* did not accumulate into microcolonies on a saliva-coated coverglass in the absence of streptococci (Fig. 2d) and no large aggregates of planktonic *P. gingivalis* cells were observed in the flow-cell inoculum. Thus, the development of *P. gingivalis* biofilms exhibits a species specificity similar to that previously observed for minor-fimbriae-mediated adhesion, suggesting that the interaction of the minor fimbriae with the appropriate antigen I/II polypeptide drives the formation of *P. gingivalis* biofilms on streptococci.

Minor-fimbriae-mediated adherence is required for *P. gingivalis* biofilm development

To further investigate the role of the interaction of Mfa1, the minor fimbrial protein, with SspB of *S. gordonii* in biofilm formation, we examined the development of biofilms on *E. faecalis* SspB, a recombinant enterococcal strain expressing SspB. As shown in Fig. 3(a), *P. gingivalis* adhered and accumulated on the *E. faecalis* SspB substratum and formed microcolonies that were similar to the structures that formed on substrates of *S. gordonii* DL1. In contrast, no accretion of *P. gingivalis* occurred on a substratum of the parent *E. faecalis* strain 401 that contains the pAM401 vector without an insert and does not express SspB (Fig. 3b).

Fig. 2. CSLM of biofilm development on saliva-coated glass in flow cells. (a) *P. gingivalis* cells (green) adhere and develop a biofilm microcolony on *S. gordonii* (red). Co-localized bacteria appear yellow. (b) *P. gingivalis* does not form a biofilm on *S. mutans* cells (red), or (c) on the Ssp-deficient mutant *S. gordonii* OB219 (red). (d) In the absence of *S. gordonii*, *P. gingivalis* cells (green) do not form biofilm microcolonies on the saliva-conditioned substratum.

To exclude the possibility that inactivation of antigen I/II expression in OB219 may also influence major-fimbriae-mediated adherence of *P. gingivalis* to *S. gordonii*, we examined the binding of purified recombinant FimA protein to OB219 cells. As shown in Fig. 4, recombinant FimA protein bound to *S. gordonii* OB219 in a dose-dependent manner and overall binding was similar to that observed with the parent organism, *S. gordonii* DL1, which expresses antigen I/II. Thus, while *S. gordonii* OB219 is capable of interacting with the major fimbriae of *P. gingivalis*, this interaction is not sufficient to induce biofilm formation. Another potentially confounding factor in interpretation of these results would be binding of SspB to additional molecules on the surface of *P. gingivalis*. To address this possi-

bility, an Mfa1-deficient mutant of *P. gingivalis* was constructed. In an interbacterial binding assay, mutant strain SMF1 demonstrated a reduction in binding to *S. gordonii* of up to 80% in comparison to the parent strain (Fig. 5). Furthermore, SMF1 was defective in biofilm formation with *S. gordonii* and with *E. faecalis* SspB (not shown). Collectively, these results support the concept that minor-fimbriae-mediated adherence is required for *P. gingivalis* biofilm formation.

BAR is sufficient to promote development of *P. gingivalis* biofilms

To confirm the role of minor fimbriae SspB-mediated adherence in biofilm development, we determined if the

Fig. 3. CSLM of biofilm development on saliva-coated glass in flow cells. (a) *P. gingivalis* cells (green/yellow) adhere and develop a biofilm microcolony on *E. faecalis* SspB (red) which expresses functional SspB protein. (b) In contrast, *P. gingivalis* does not form biofilms on *E. faecalis* 401 (red) which does not express SspB.

Fig. 4. ELISA of recombinant fimbriin binding to *S. gordonii* strains DL1 (black bars) or OB219 (white bars). Error bars represent standard deviation ($n=3$).

Fig. 5. Adhesion of *P. gingivalis* 33277 (●) and its minor-fimbriae-deficient mutant, SMF1 (■), to cells of *S. gordonii* DL1. Error bars represent standard deviation ($n=3$).

BAR region of SspB was sufficient to promote the development of *P. gingivalis* biofilms. To accomplish this, we monitored the formation of *P. gingivalis* biofilms on *E. faecalis* strains expressing chimeric SspB/SpaP polypeptides and on strains expressing SspB that contained site-specific mutations of functional amino acid residues of BAR. Chimeric proteins Spac4 and Spac5 (see Methods) contain residues 1–1167 and 1–1250 of SspB, respectively, fused to the appropriate C-terminal sequences of SpaP. Thus, Spac5 differs from Spac4 only in that it contains BAR. As shown in

Fig. 6(a), *P. gingivalis* accumulated on immobilized *E. faecalis* Spac5 cells, forming microcolonies similar in structure and depth to those observed on *E. faecalis* SspB and on *S. gordonii* DL1. In contrast, *P. gingivalis* cells did not accumulate on *E. faecalis* Spac4 (Fig. 6b) or on *E. faecalis* strains expressing SspB polypeptides in which Asn¹¹⁸² and Val¹¹⁸⁵ were substituted with the corresponding residues of SpaP, Gly¹¹⁸² and Pro¹¹⁸⁵ (not shown). These results show that *P. gingivalis* biofilms do not form in the absence of the SspB BAR domain. In addition, when specific amino acid residues of the BAR

Fig. 6. CSLM of biofilm development on saliva-coated glass in flow cells. (a) *P. gingivalis* cells (green/yellow) adhere and develop a biofilm microcolony on *E. faecalis* Spac5 (red) which expresses a chimeric SspB/SpaP polypeptide possessing the BAR domain. (b) *P. gingivalis* cells do not develop into biofilms on *E. faecalis* Spac4 (red) which expresses a chimeric SspB/SpaP polypeptide that does not possess the BAR domain.

region of SspB are altered to the corresponding residues of the *S. mutans* SpaP polypeptide, then biofilm formation is impeded.

DISCUSSION

The human oral cavity represents a hostile environment for micro-organisms due to the presence of multiple antimicrobial activities and the constant flow of saliva across tissue surfaces (Marsh, 1989; Rosan & Lamont, 2000; Scannapieco, 1994). Oral bacteria insulate themselves from these adverse conditions by adhering tightly to oral tissues and to other oral organisms to form a complex multispecies biofilm. The initial colonization of the plaque biofilm by *P. gingivalis* may thus be facilitated by its adhesion to other bacteria such as *F. nucleatum* (Bradshaw *et al.*, 1998; Kinder & Holt, 1989; Kolenbrander & Andersen, 1989) and specific oral streptococci such as *S. gordonii* (Lamont *et al.*, 1992). However, the initial attachment to antecedent bacteria in plaque represents only the first step in the process by which *P. gingivalis* becomes established and persists in the oral ecosystem. The subsequent expansion of *P. gingivalis* populations in plaque is likely to be dependent upon nutritional and environmental factors (Guggenheim *et al.*, 2001; Marsh, 1994) along with the ability of adherent *P. gingivalis* to recruit additional planktonic cells into the developing sessile biofilm (Cook *et al.*, 1998).

Our studies have focused on the adherence of *P. gingivalis* to streptococci and the subsequent formation of biofilms on the streptococcal substrate. Adherence of *P. gingivalis* is mediated by at least two distinct

adhesin–receptor pairs (Brooks *et al.*, 1997; Chung *et al.*, 2000; Lamont *et al.*, 1993) involving both the FimA and Mfa1 proteins, the predominant polypeptides composing the major and minor fimbriae of *P. gingivalis*, respectively. Both adhesins have been shown to mediate adherence to *S. gordonii* under static conditions *in vitro*. Several lines of evidence from the present investigation suggest that the development of *P. gingivalis* biofilms on a *S. gordonii* substratum is dependent primarily on the interaction of minor fimbriae with streptococcal antigen I/II. First, the development of *P. gingivalis* biofilms exhibited the same species specificity as was previously demonstrated for minor-fimbriae-mediated adherence to streptococci *in vitro*. Second, biofilms did not develop on *S. gordonii* OB219, a strain that does not express the antigen I/II receptor for the minor fimbrial adhesin but which retains the ability to interact with the major fimbrial adhesin. Thus, FimA-mediated adhesion alone is insufficient to induce the formation of biofilms. Third, a minor-fimbriae-deficient mutant of *P. gingivalis* was impaired in its ability to bind to *S. gordonii* and was unable to form biofilms. The structural motif (BAR) of antigen I/II that is recognized by Mfa1 also was shown to be essential for the development of *P. gingivalis* biofilms. Recombinant *E. faecalis* strains expressing chimeric SspB/SpaP proteins lacking BAR or expressing SspB site-specific mutants lacking essential residues of BAR did not support biofilm development. These results demonstrate that wild-type *P. gingivalis* (FimA⁺, Mfa1⁺) are unable to form biofilms on streptococci or enterococcal constructs that do not possess the appropriate antigen I/II determinants, even if the cells are capable of interacting with FimA. Thus, individual components of the multimodal binding interaction appear to have

Fig. 7. Schematic model of events contributing to the formation of a *P. gingivalis* biofilm on immobilized *S. gordonii* cells. (a) The initial association of *P. gingivalis* with *S. gordonii* is mediated by the interaction of the major fimbriae, comprised of the FimA protein (●) with an unidentified receptor on the *S. gordonii* cell surface (■). (b) Higher-affinity binding of *P. gingivalis* to *S. gordonii* occurs as FimA and Mfa1 (○) engage their cognate receptors. Mfa1 interactions with SspB (□) induce signalling events (→) within *P. gingivalis* that lead to phenotypic changes, potentially alteration of surface receptors (<) and/or expression of extracellular signalling molecules (◇). (c) Planktonic cells adhere to the immobilized *P. gingivalis* cells and accrete into a biofilm microcolony.

discrete and independent roles. Consistent with this concept, Love *et al.* (2000) found FimA binding to *S. gordonii* to be independent of SspA or SspB and that a FimA mutant was able to co-invade dentinal tubules with *S. gordonii*.

While our results indicate that the Mfa1–SspB-mediated interaction is necessary for biofilm development, they do not completely exclude the possibility of a role for the other adhesin–receptor pairs that participate in the multimodal *P. gingivalis*–*S. gordonii* coadhesion event. These molecules could serve either to allow initial low-affinity contact between the organisms or to help stabilize the interaction between adhered organisms. The long major fimbriae that can extend up to 3 µm from the cell surface would be suitable candidates to effect relatively long-range, low-affinity interactions with the streptococci. The proximal association between the two organisms would then allow the close range, higher affinity Mfa1–SspB interactions that induce biofilm formation. The role of the major fimbriae will be

more apparent when the identification and distribution of their cognate receptor is accomplished. This model of *P. gingivalis*–*S. gordonii* coadhesion and biofilm development is represented in Fig. 7. Longer distance interactions, primarily mediated by the major fimbriae, facilitate localization of cells of *P. gingivalis* and *S. gordonii*. Multimodal coadhesion then occurs involving both Mfa1–SspB and FimA–receptor interactions. The engagement of Mfa1 with SspB initiates a signal transduction event within *P. gingivalis* that results in cells that are primed for biofilm formation. Theoretically, the transformation to a biofilm ready state could entail expression of a surface receptor that permits autoaggregation of *P. gingivalis* and stimulates other *P. gingivalis* cells to produce the same receptor. Alternatively, or additionally, a soluble signalling molecule may be released by *P. gingivalis* that stimulates cells to accumulate on the solid phase.

Biofilm formation by other organisms such as *Pseudomonas aeruginosa* requires cell-to-cell signalling through quorum sensing (Davies *et al.*, 1998). *P. gingivalis* does not possess homoserine lactone-based signalling systems that are used by *Pseudomonas aeruginosa* for biofilm differentiation; however, *P. gingivalis* does have a functional LuxS-based quorum sensing system (Chung *et al.*, 2001). The role of LuxS in *P. gingivalis* biofilms remains to be determined as does the possibility that signalling molecules produced by the streptococci or by *E. faecalis* are also involved in the biofilm process.

The maintenance of the complex oral biofilm community is thought to depend on a series of cohesive interactions that stabilize plaque structure and facilitate nutritional interrelationships (Kolenbrander & London, 1993; Rosan & Lamont, 2000). Moreover, the adherence event itself can transduce information into the bacterial cell regarding the substrate and environment in which the organism has localized (Cleary & Retnoningrum, 1994; Cornelis & Van Gijsegem, 2000; McNab & Jenkinson, 1998). In the case of BAR-mediated adhesion, *P. gingivalis* responds by accretion into microcolonies. This process can be postulated to involve intracellular signalling pathways within *P. gingivalis*, an example of which has recently been described (Hayashi *et al.*, 2000), and modulation of gene expression. The complete range of phenotypic changes that accompany biofilm formation remains to be determined.

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